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Fermentation of Carcinus Maenas as a Method of Waste Stream Valorization

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FERMENTATION OF *CARCINUS MAENAS* AS A METHOD

OF WASTE STREAM VALORIZATION

By

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An Abstract of the Thesis Presented
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The European green crab, *Carcinus maenas*, is an aggressive invasive species of green crab that has had severe ecological and economic effects to the North American East Coast since the early 1800s. The green crab has disrupted entire ecosystems, particularly in the Gulf of Maine, due to its eating and uprooting of eel grass. The disruption of this plant reduces coastal habitats for bivalves and other coastal marine life. The green crabs have also been found to prey on juvenile lobsters and bivalve mollusks, disrupting both the ecosystem in the Gulf of Maine and the seafood economy dependent on lobsters and soft-shell clams.

With the changing climate and consistent increases in the ocean temperature, the number of crabs killed by the cold, winter temperatures has been steadily decreasing. The establishment of a green crab fishery for the sale of soft-shell crabs has been identified as a method to control the growing population since the crabs are too small for hard-shell culinary use. However, most crabs fished cannot be sold as soft-shell, creating a large amount of biomass. The biomass can currently only be sold to low-valorization waste streams such as compost, bait, or animal feed. In order to sustain green crab fisheries, a high-value waste stream valorization needs to be established.

Fish sauce is a clear, brown, fish-flavored condiment that is traditionally spontaneously fermented from under-utilized fish species and salt. Fish sauce originated in Asia, but has been fermented in many places throughout the world including Rome, South America, and Africa with slight variations to the formulation depending on location and starting material. Since this fermentation is spontaneous it relies on the proteolytic activity of endogenous enzymes and proteolytic bacteria to break down proteins into amino acids. One of the most common fish species used for the fermentation of fish sauce is the anchovy, which has a protein content of about 20%. Green crabs have a protein content of about 17%, indicating that green crabs could be used instead of anchovies to produce a similar condiment.

The data from this work showed that when fermented at a temperature of 24°C at a salt content of 20-30%, a green crab fermented condiment can be produced that is chemically comparable to commercially available fish sauce products. Fermentation temperatures of 30°C, 37°C, and 50°C were investigated to provide guidance for temperature control. A temperature of 30-37°C with a salt content of 20% and a 90 day fermentation time was suggested based on this data, but a temperature of 24°C or 50°C will still produce a viable product. The most abundant families of bacteria throughout the course of the fermentation, regardless of fermentation temperature or time, were *Rhodobacteracea, Saprospiraceae, and Hyphomonadaceae*, all of which contain salt-tolerant proteolytic genera isolated from marine sources. The creation of a viable fermented crab sauce product creates a high-value waste stream for green crab fishers, thus opening the doors to start economical large-scale fishing of green crabs on the North American East Coast.

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LITERATURE REVIEW

1.1. Introduction

Carcinus maenas, an invasive species of green crab, was introduced to the North American East Coast in the early 1800s (Klassen & Locke, 2007). Its ever-increasing population has taken a tremendous toll on the economic growth and ecological systems in the marine environment. There are options to control the growing population, but limited options geared towards exploitation for financial gains. The green crabs are relatively small, making the meat very difficult to extract, so there is only a soft-shelled industry for the green crabs (McNiven et al., 2013), but large proportions of biomass waste is produced. Currently, there are no high-value options for this biomass waste, only two low-value options – animal feed or compost.

The popularity of fish sauce throughout the world, both in culinary and home use, creates an opportunity to exploit fermented green crab sauce as an option for consumers. Fermentation of the invasive species to make a fermented crab condiment could be a viable option to create an economic opportunity and incentivize fishers to catch this species. In the long run, this may reduce the negative effects that the growing green crab population has on the economy and ecology of marine environments.

The development of food products encompasses a thorough evaluation of several factors to help meet all necessary sensory and safety measures to ensure FDA compliance and consumer acceptability. Although fermenting the green crabs is a seemingly straightforward idea, there are considerations vital to the safe and effective fermentation of such a product. This research seeks to address the safety, microbial, and chemical composition of a novel fermented green crab condiment.

This literature review introduces the species *Carcinus maenas*, discusses efforts to control the species, and introduces fish sauce fermentation.

1.2. Invasive Species

Invasive species are organisms that can thrive and cause harm in ecosystems to which they are not native through their ability to compete with native species for resources. Species with this ability outcompete native species in their existing environments, causing population decline and sometimes even the extinction of native species.

The resilience and growth of invasive species can cause both ecological and economic harm. Invasive species exist all over the earth. Specifically, in the United States, about 50,000 invasive species have been introduced (Pimentel et al., 2005). The introduction of invasive species has occurred on land and by sea. The introduction of invasive species by land is more easily controlled due to the limited range and dispersal method. Introduction of invasive species through waterbodies, particularly oceans, is more difficult to prevent due to the natural range expansion of human-mediated introductions, and often occur through intentional release or accidental release from the ballast water of ships (Carlton, 1996). Additionally, the interconnection of all oceans results in the broader distribution of invasive species around the globe.

1.2.1. Native and Invasive Crabs on the North American East Coast

There are a few commercially important species of crab that are fished on the North American East Coast, including but not limited to blue crab, Jonah crab, red deep-sea crab, and Norway king crab. These species already serve the existing market and are notably larger than the green crab (Table 1.1).

Table 1.1. Native and Invasive Crabs on the North American East Coast			
Species	**Common Name**	**Maximum Carapace Width**	**Source**
Callinectes sapidus[a]	Blue Crab	127 mm	NOAA Fisheries, 2021b
Carcinus maenas[b]	Green Crab	79.7 mm	Skonberg & Perkins, 2002
Cancer borealis[a]	Jonah Crab	63.2-158.7 mm	Truesdale, 2018
Chaceon quinquedens[a]	Red Deep Sea Crab	114-128mm	Trigg & Perry, 1997
Lithodes maja[a]	Norway King Crab	76.8-96.7mm[c]	DFO Stock Status Report, 1998

[a]Designates native, commercially important species
[b]Designates invasive species
[c]The carapace width of these crabs is small, but commercially they are harvested for their leg meat.

Compared to crab species that are commercially important to the North American East Coast, green crabs have a small carapace width. The only species that have a comparable carapace width are the Norway king crab, which is harvested for its leg meat (DFO Stock Status Report, 1998), and the Jonah crab which can only be commercially harvested when the carapace width is 120 mm or larger (National Ocean and Atmospheric Administration, 2021a). Since the green crab is much smaller than the other commercially important crab species, and the texture of the cooked meat is softer than most commercially relevant crabs, it cannot be profitably picked for meat and the only commercially relevant application would be soft-shell crab products.

Interestingly, it has been recorded that the overall nutritional composition of green crab is dependent on its size rather than the time of harvest. Therefore, there is no ideal season or time for harvesting (McNiven et al., 2013). Hence, a perfect catch relies heavily on size, which is the primary determinant of the harvested crab's nutritional composition.

1.2.1.1. Green Crab Origins and Characteristics

European Green Shore Crab (*Carcinus maenas*), hereafter referred to as “green crab,” is an invasive species on the North American East Coast first seen in North America in 1817, off the coast of Massachusetts (Klassen & Locke, 2007). The green crab is originally from Europe and North Africa but has invaded both the Atlantic and Pacific coasts of all continents except Antarctica (Klassen & Locke, 2007).

Green crabs have an expected lifespan of about 4-7 years and thrive in the intertidal zone, where the land meets the ocean between the tides (Berrill, 1982; National Ocean and Atmospheric Administration, 2021b) and prefer dwellings in salt marshes and seagrass beds. Green crabs move into deeper water during the colder months, creating a reduction in activity (Grosholz, E., Ruiz, 2002) due to the inability to grow at temperature below 7-10°C (Berrill, 1982). The green crab is distinguished by its fan-like carapace, five anterior-lateral teeth behind the eyes, and three lobes between the eye sockets, as shown in Figure 1.1. (Yamada & Hauck, 2001).

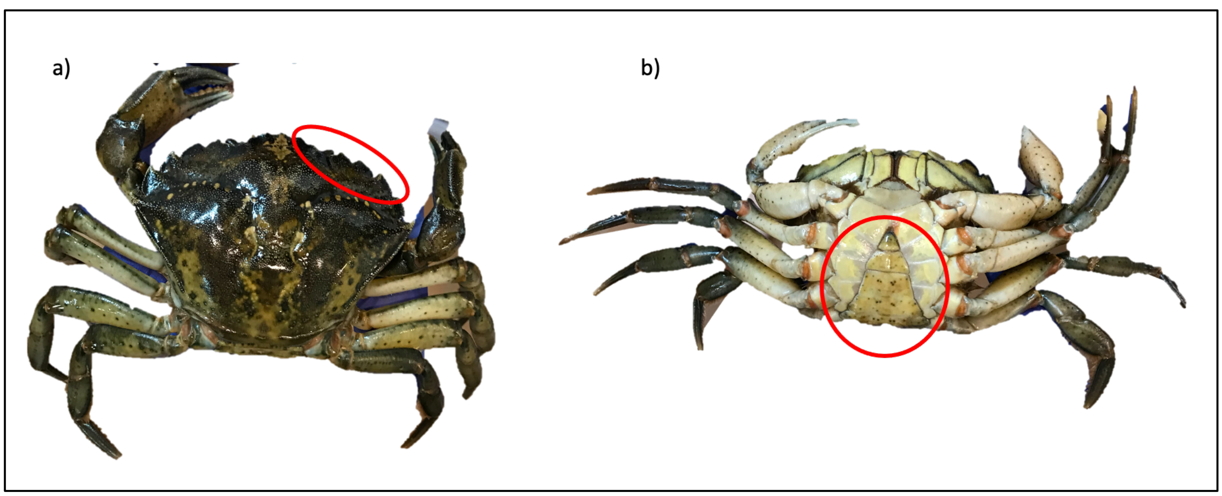

Figure 1.1. Identification of European Green Crabs. a) A green crab is identified by the anterio-lateral teeth behind its eyes (circled in red). b) The gender of green crabs can be determined by the shape of the underside. The males have a more pointed shape (circled in red) and the females have a more rounded shape.

1.2.1.2. Ecological Effects of Green Crab

Green crabs cause ecological problems both for the native flora and fauna due to predation of native species, such as soft-shell clams and American lobster, as well as the destruction of eelgrass beds. Green crabs' ability to outcompete almost any predator (Matheson & Gagnon, 2012) has adversely affected American lobsters on the North American East Coast. Not only do green crabs get to food significantly faster, but succeed in defending the food from heavier lobsters (Williams et al., 2006). Even when American lobsters were allowed to take control of the food source first, green crabs physically overpowered juvenile lobsters because of their agility and aggression (Williams et al., 2006). The green crabs' ability to outcompete native species was consistent even when subjected to different temperatures (Matheson & Gagnon, 2012).

Green crabs have drastically affected the native ecology of the North American East Coast due to their predation on native species. Green crabs have been found to prey on barnacles, gastropods, marsh grasses, arthropods, insects, and bivalve mollusks (Congleton et al., 2006; Rangeley & Thomas, 1987; Tyrrell et al., 2006). Dietary habits change with the crab's age, with juvenile crabs being drawn to grasses and soft-shelled animals and adult crabs preying on mollusks (Rangeley & Thomas, 1987).

Bivalve mollusks are the most preferred prey for green crabs, resulting in other ecosystem changes (Grosholz & Ruiz, 1996). The declining population of softshell clams has dramatically decreased landings on the Maine coast due to the predation by green crabs and constant fishing pressure (Congleton et al., 2006). Aiding in the green crab’s impact on the clam population is the intense change of climate in the Gulf of Maine. The sea surface temperature in the Gulf of Maine has increased by as much as 0.4°C per decade (Thomas et al., 2017). The amount of winterkill in green crabs is inversely correlated to ocean temperatures, allowing the green crab population to grow and increasingly prey on *Mya arenaria*, the soft shell clam (Congleton et al., 2006; Klassen & Locke, 2007). The clams have suffered massive decreases in population because of the impact of green crabs on both commercial and non-significant species (Congleton et al., 2006; Department of Fisheries and Oceans Canada, 2011).

Based on evidence from the Department of Fisheries and Oceans Canada (2011), there is also concern for predation of green crab on both adult and juvenile lobsters. The American Lobster only thrives in the North to Mid Atlantic, a very small geographic range, and is highly regulated by the Maine Department of Marine Resources (Maine Department of Marine Resources, 2021b; NOAA Fisheries, 2021a) due to the high value of the species. In 2020, the Maine lobster industry landings were valued at $408 million (Maine Department of Marine Resources, 2021a).

In addition to predation on the native fauna, green crabs have been responsible for the disruption of native flora. Green crabs primarily cause damage by uprooting large amounts of eelgrass. Full-sized green crabs have the potential to uproot an estimated 84% of available eelgrass shoots (Malyshev & Quijón, 2011). In most cases, the green crabs do not eat the eelgrass but are burrowing for shelter and digging for prey within eelgrass beds (Department of Fisheries and Oceans Canada, 2011). This burrowing and digging uproots the eelgrass or exposes and weakens the roots (Malyshev & Quijón, 2011). The green crabs tear or cut the transplanted grass with their typical burrowing and foraging behavior (Davis et al., 1998). In addition to the disruption of the eelgrass beds, the green crabs also disrupt entire ecosystems that depend on the eelgrass for either food or shelter. Therefore, reducing the population of green crabs would be the most effective way of restoring the ecosystem since the disruption of the eelgrass population along the east coast of North America is directly attributed to green crabs.

1.2.1.3. Economic Effects of Green Crab

Whether introduced to the land or sea, Pimentel et al. (2005) have estimated that invasive species' control has cost almost $120 billion per year in the United States. The presence of green crabs has upset the economy of a particular industry in New England. The shellfish culture and fishing industries have specifically suffered the most economic loss, and the eel fisheries have struggled as well (Klassen & Locke, 2007). The portion of this sector that has suffered the most intense economic loss has been the soft-shell clam fishery. The soft shell clam is a large industry for Maine, maintaining a value of $15 million in 2020 (Maine Department of Marine Resources, 2021a).

1.2.2. Measures to Control Green Crab in New England

Although green crabs have already undergone an intense population growth off the coast of New England, research indicates that the distribution is dramatic on both the North American East and West Coasts (Grosholz & Ruiz, 2002). This non-native species poses a threat to native ecosystems and existing marine economies, but population control requires a large financial input, which is not typically available (Pasko & Goldberg, 2014). When a green crab is identified in a new location, timing is everything. Its identity, source, and mode of introduction need to be identified. Once these are determined, concrete steps can be taken to prevent an increase of an invasive population (Yamada & Hauck, 2001).

Intense trapping appears to be an effective control method for the mitigation of green crab populations (Department of Fisheries and Oceans Canada, 2011). The establishment of green crab fisheries has been supported heavily to control this invasive species, due to the success of analogous fisheries, internationally, and the low starting cost allowed by use of existing and readily available materials (Skonberg & Perkins, 2002). Although trapping tends to be a popular choice in controlling green crab populations, there are hurdles to this method including the limitations of a soft-shell industry and need for waste stream valorization.

1.2.2.1. Green Crab Fisheries

Fisheries tend to be one of the most uncomplicated control measures to implement with a relatively low starting cost, because materials used for other activities can be utilized for green crab fishing. Green crabs reproduce readily and maintain high population levels. Trapping these green crabs will reduce the average size of the green crab population over time, and trapping large crabs would shift the green crab's position in the food chain (Department of Fisheries and Oceans Canada, 2011).

The season for green crab trapping would not need to be limited to a short period, creating an even better opportunity to implement a fishery. Due to the constant chemical composition of green grabs over a six month season, green crabs don’t have an optimal harvest period, creating opportunities to use existing seafood processing plants (McNiven et al., 2013). Although there is a long season for green crabs, specifically trapping them at the beginning of the season, before propagating, would reduce the impact of the crabs on the environment (McNiven et al., 2013).

Currently in Maine, soft-shell green crabs can be sold directly to restaurants for $25/lb (McMahan, 2021), with evidence that fisheries have the potential to reach approximately $60/lb (Associazioni di consumatori iscritte nel Registro Regionale delle Associazioni dei Consumatori e degli Utenti - anno 2014 della Regione Veneto, 2014 as cited in St-Hilaire et al., 2016). This existing high-value fishery in Italy serves as an example of a thriving fishery that could be established on the North American East Coast. Manomet, a non-profit that engages coastal communities to reduce pressure on habitat and impacts of climate change (Manomet, 2021), has been hosting virtual seminars during the Covid-19 pandemic that show those involved in the fishing industry how to create an economic opportunity by using materials they already own to trap and store green crabs. These opportunities seem very lucrative to pursue opening a green crab fishery on the east coast of the United States, and if a fishery is successfully established, it could be a viable option to control, but not completely eradicate the green crab population (St-Hilaire et al., 2016).

1.2.2.2. Waste Valorization

The most natural inclination to valorize animal products is through culinary application. However, since this species is quite undersized compared to other commercially available crab species, green crabs cannot be easily picked for meat but only prepared as soft-shell, or used as a flavoring agent in compound dishes. Such inclusion of green crab in culinary applications includes deboning of the crabs or use in stocks. Previous research has indicated that culinary uses of deboned green crab mince shows promise in culinary applications (Galetti et al., 2017). Soft-shell green crab has started to be widely used in Maine as a culinary trend, most publicly at the Brunswick Inn by Chef Ali Waks-Adams (Clemente, 2018). Since trending in restaurants, these crabs have also been welcomed into home-cooking with The Green Crab Cookbook (Green Crab R&D, 2017).

Since the culinary industry currently only has a use for the soft-shell product, valorization of crabs unsuitable for this use is imperative. A low-value option is to incorporate them into animal feed. Unfortunately, the ash content of the whole product is a limiting factor when using the green crabs in animal feed as a primary ingredient (McNiven et al., 2013). There is a need for a valorization of these hard-shell and low quality soft-shell green crabs into a more profitable product.

1.3. Fish Sauce

Fish sauce is a fermented, brown, translucent liquid with a strong salty fish flavor made from fish and salt (Food and Agriculture Organization of the United Nations, 2012). Fish sauce has a salt concentration of 15-25%, which typically results in use as a condiment (El Sheikha & Montet, 2014). Fish sauce, although smelling fishy, adds much salt and a rich umami flavor to dishes. Fish sauce is commonly used in pad Thai or stir fry but is also incorporated into salad dressings, pasta sauces, marinades, and soups (Beggs, 2020). In recent history, fish sauce has been increasingly consumed all over the world. As of 2011, 43 million liters of fish sauce were exported from Thailand, 22% of which went to the United States (Food and Agriculture Organization of the United Nations, 2012).

1.3.1. History of Fish Sauce

Fish sauce fermentation relies on the conversion of low-value fish into a high-value sauce to enhance the economic value of the fish and reduce the environmental impact of fishing practices (Zhou et al., 2016). Fish sauce fermentation is a fundamental method of preservation because it uses low-quality fish, fishery byproducts or unpopular species. This product is traditionally associated with Asian cuisines, but has evolved worldwide due to the easy and low cost fermentation process involved (El Sheikha & Montet, 2014). This adaptation and popularity of fish in many different places led to many different fermented fish sauce products created worldwide (Table 1.2.).

Table 1.2. Fish Sauce Products Around the World		
Country of Origin	**Fish Sauce Product**	**Defining Characteristics**
Japan	Pla ra	Fermented from sandfish, sardines and squid Malted rice and koji commonly added
	Uwo-shoytu	
	Ikashoyu	
Cambodia	Nuoc-mam	Ferments 3-12 months with 3:1[a]
Thailand	Nam-pla	Ferments 5-12 months with 5:1[a]
Malaysia	Budu	Includes palm sugar and tamarind
Phillippines	Patis	Ferments 3-12 months with 4:1[a]
Indonesia	Ketjap-ikan	Ferments for 6 months with 6:1[a]
	Bakasang	Ferments 3-6 weeks
India and Pakistan	Colomba cure	Remove guts and gills, add tamarind
Ghana	Momoni	Ferments 1-5 days with 10:3[a]
Greece	Gaross	Contains only liver Ferments for 8 days
France	Pissala	Ferments 2-8 weeks with 4:1[a]
	Anchovy	Uses beheaded and gutted fish

Adapted from Fish Sauces by El Sheikha &Montet. 2014.
[a]Fish:Salt ratio

Other countries that produce fish sauce or similar products are Egypt, Sudan, South Korea, and Myanmar. A fermented crab meal condiment, ogiri-nsiko, is also popular in West Africa. This product is made from a freshwater crab and is primarily used in soup preparation (Achi et al., 2007). Despite the popularity of fish sauce in the modern American diet, there is no documentation in the literature of any commercially available seafood sauces produced from a domestic fishery.

1.3.2. Composition of Fish Sauce

Inconsistencies in the production of fish sauce may result in a difference in the finished product's chemical composition, which affects sensory characteristics (Park et al., 2001). These compositional differences depend on fish species, the type of salt, the ratio of salt and fish, the addition of non-traditional ingredients, and the conditions of fermentation (Tanasupawat & Visessanguan, 2014). Despite compositional differences, specific standards exist that products sold as fish sauce should adhere to specifications set forth by the Food and Agriculture Organization of the United Nations and the World Health Organization (Table 1.3.).

Table 1.3. Standard Chemical Properties of Fish Sauce

Total Nitrogen	Not less than 10 g/L[a]
Amino Acid Nitrogen Content	Not less than 40% of Total Nitrogen
pH	Between 5.6-6.5[b]
Salt	Not less than 200 g/L[c]

[a] "Competent authorities may also specify a lower level of total nitrogen if it is the preference of that country." (Food and Agriculture Organization of the United Nations & World Health Organization, 2011)
[b] "Not lower than 4.5 if ingredients are used to assist fermentation." (Food and Agriculture Organization of the United Nations & World Health Organization, 2011)
[c] "Calculated as NaCl" (Food and Agriculture Organization of the United Nations & World Health Organization, 2011)

The flavor and aroma of fish sauce are also central to the identity of the product. Fish sauce provides a very distinct and rich umami taste that is stronger than soy sauce (Park et al., 2001). An integral part of this rich umami flavor is the presence of glutamic acid, released during fish sauce fermentation (Mizutani et al., 1992). Fish sauce products are also generally associated with notes that are described as fishy, nutty, meaty, or cheesy (Giri et al., 2010).

1.3.3. Modern Culinary Uses of Fermented Foods

Fermented foods have become increasingly popular in a modern diet based on perceived health benefits and are being demanded by consumers (Dimidi et al., 2019). The incorporation of fermented products is trending in both small quaint restaurants as well as fine-dining dynasties. The most well-known restaurant heavily incorporating fermented products is Noma, in Copenhagen, Denmark. This restaurant uses at least seven types of fermented foods or sauces at any given time to introduce several unique flavors that improve all dishes the restaurant offers (Redzepi & Silber, 2018). The culinary staff started incorporating these fermented products, and they have since created a separate fermentation kitchen to create new flavors and products consistently. The executive chef and lead fermentation scientist have published *The Noma Guide to Fermentation* to aid home cooks in incorporating the same flavors at home.

Knowledge for home-fermented products is also very widely available. Sandor Katz, a fermentation revivalist, has created a platform to promote home fermentation as not only a form of honoring ancestral recipes, but also as a form of activism (Vaughan-Lee, 2020). This fermentation guru has identified home fermenting as a way to fight food insecurity and shares his information through multiple books, the most notable being *The Art of Fermentation*, a New York Times Best-seller.

1.3.4. Fish Sauce Fermentation

A typical fermentation process is when foods are “spoiled” in a controlled way or manipulated by microorganisms (Emerald et al., 2016). This process can be designed using starter cultures or as a spontaneous fermentation. Fish sauce is traditionally produced using spontaneous fermentation, where the starting material is degraded by naturally contained enzymes and microorganisms (El Sheikha & Montet, 2014).

As a spontaneous fermentation, the transformation of raw materials during the course of fish sauce fermentation is primarily due to the action of endogenous proteases and exogenous microbial proteolytic enzymes. These enzymes, which come from the digestive system of the fermented material, in this case fish, and microbes, break proteins into a collection of nitrogenous compounds (Martínez-Álvarez et al., 1967). Salt is included for flavor and to provide an ideal environment for the growth of halophilic bacteria (Lopetcharat et al., 2001), typically responsible for fish sauce fermentation. The resulting product is yellow or brown, salty, and has a fishy aroma after a 12-18 month fermentation (Tanasupawat & Visessanguan, 2014).

1.3.4.1. Microbial Activity

The spontaneous fermentation and inclusion of salt favor the growth of halotolerant and halophilic microorganisms (Jiang et al., 2007) present on the starting materials. Lactic acid bacteria, yeasts, and molds, although often utilized in both inoculated and spontaneous food fermentations, are not typically responsible for the spontaneous fermentation of fish sauce despite being present on starting materials (Kilinc et al., 2006) due to low salt tolerance.

The genus *Bacillus* has been identified as the primary genus responsible for the fermentation and flavor development in fermented fish sauce (Xiao et al., 2014). In addition to their ability to form spores, *Bacillus* can also produce a variety of enzymes imperative to flavor development and amino acid degradation (Achi et al., 2007). Other species that have been identified to be present in fish sauce include *Micrococcus luteus* BML1, *Staphylococcus xylosus, Chromohalobacter zalexigens, Halobacterium salinarum,* and *Halobacterium saccharolyticus.* (Sim et al., 2012; Tanasupawat et al., 2008). Despite the lack of lactic acid bacteria in traditional spontaneous fish sauce fermentation, evidence suggests that lactic acid bacteria could be useful in preventing the development of undesirable fecal notes (Tanasupawat & Visessanguan, 2014).

1.3.4.2. Biogenic Amine Formation

As discussed previously, fish sauce production is primarily related to the enzymatic degradation of seafood proteins. When the fish's proteins are broken down rapidly, the enzymes can generate biogenic amines (Figure 1.2; Zhai et al., 2012), which are organic bases with a low molecular weight that are associated with high biological activity (Zhou et al., 2016).

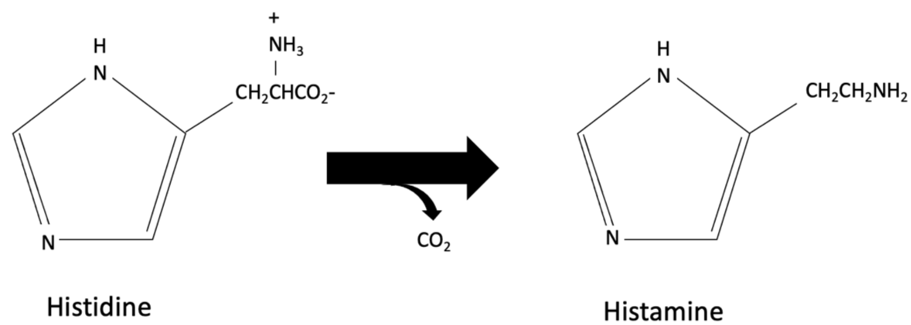

Figure 1.2. Histamine Formation due to the Enzyme Histidine Decarboxylase. Based on a figure from (Amani et al., 2018).

In many food systems, biogenic amines are mainly produced by *Enterobacteriaceae* and enterococci (Nout, 1994), but more recent research has identified aerobic and proteolytic bacteria as responsible for biogenic amine production as well (Zaman et al., 2011).

Histamine poisoning, also known as scombroid poisoning, is a significant illness caused by high levels of the biogenic amine histamine (Lipp & Rose, 1997). The symptoms of histamine poisoning closely resemble those of an allergic reaction. Histamine poisoning is typically identified by symptoms such as headache, nausea, vomiting, diarrhea, and a burning sensation in the mouth and throat (Lipp & Rose, 1997; Sánchez-Guerrero et al., 1997). Histamine poisoning has relatively short periods of incubation that can range from only minutes to hours (Sánchez-Guerrero et al., 1997). Due to these potential food safety problems with biogenic amine production, fish sauce products should be carefully monitored to ensure safety (Zhai et al., 2012).

Biogenic amines, also associated with wine, cheese, and meat (Nout, 1994), can be controlled by making the fermentation shorter, but this could result in a fermented product that has not been fully fermented, whose flavor is undeveloped (Zhou et al., 2016). The inclusion of starter cultures provides an opportunity to control biogenic amine production over the course of the fermentation.

1.3.5. Starter Cultures

Starter cultures have become the main form of producing consistent fermented products (El Sheikha & Montet, 2014). These can be a high population of one or multiple microorganisms that are meant to guide and potentially accelerate the fermentation process (Saithong et al., 2010). Starter cultures can even keep the food safe during fish sauce fermentation. Starter cultures can be used in the form of yeasts, molds, and bacteria. Although not typically used in fish sauce fermentation, starter cultures have been found to effectively reduce biogenic amine accumulation in fish sauce (Zaman et al., 2011).

1.3.5.1. Bacterial Starter Cultures

Bacteria with amine oxidase activity (Figure 1.3) have been of primary interest when identifying starter cultures that could reduce biogenic amine levels in fish sauce fermentation (Zaman et al., 2011).

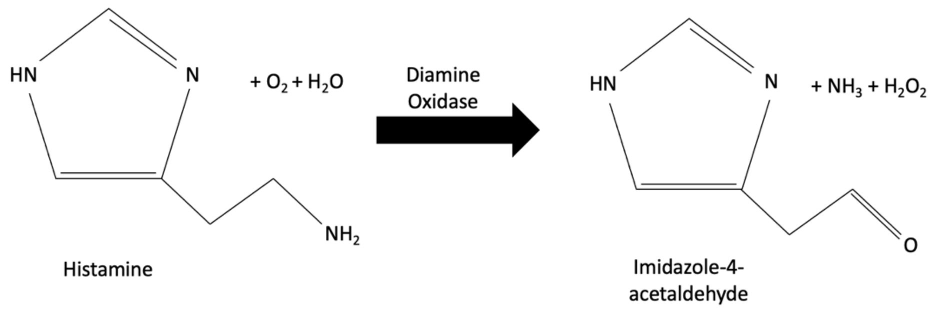

Figure 1.3. Oxidative Deamination of Histamine. Based on a figure from (Comas-Basté et al., 2021).

Several bacterial starter cultures have been identified as histamine degrading (Dapkevicius et al., 2000). These cultures include *Bacillus amyloliquefaciens* FS-05, *Natrinema gari,* and *Staphylococcus xylosus* (Mah & Hwang, 2009; Tapingkae et al., 2010; Zaman et al., 2011). In order to prevent histamine from forming, starter cultures with the amine oxidase capability should be added at the beginning of the fermentation to prevent biogenic amines from accumulating instead of degrading existing biogenic amines as well as outcompeting biogenic amine producing bacteria (Zaman et al., 2011). Although there are many starter cultures to choose from, the most appealing are *Staphylococcus carnosus* and *Tetragenococcus halophilus* due to their activity at high salt concentration.

1.3.5.1.1. *Staphylococcus carnosus*

Staphylococcus carnosus is a Gram-positive coccus that is accepted as a halotolerant microorganism capable of degrading histamine (Zaman et al., 2014). *S. carnosus* grows best at 35°C, a pH of 8, and with a salt content of 9%. When used in a fermented fish sauce for 120 days, *S. carnosus* resulted in a lower accumulation of histamine (-15.9%), but may not prevent the accumulation of histamine at a salt content greater than 21% (Zaman et al., 2014).

1.3.5.1.2. *Tetragenococcus halophilus*

Tetragenococcus halophilus is a non-spore forming coccus that is non-motile and Gram-positive (Justé et al., 2011). Inclusion of this starter culture in a fish sauce fermentation significantly reduced histamine concentration and dimethyl disulfide, a compound contributing to fecal aroma notes (Udomsil et al., 2011). *T. halophilus* effectively reduced the concentration of biogenic amines throughout fermentation, but this bacterium has also positively impacted volatile compound formation during fish sauce fermentation (Tanasupawat & Visessanguan, 2014).

1.3.5.2. Fungal Starter Cultures

Mold starter cultures are an option for keeping biogenic amine contents low even though they are an atypical component of spontaneous fish sauce fermentation. Koji is a mold cultured on soybean typically used in the fermentation of soy sauce, miso, and sake (Chisti, 2014). Mixed kojis have been investigated as an accelerant of the fermentation process, as a way to prevent formation of biogenic amines, and a way to develop a more intense umami flavor (Zhao et al., 2017). When testing consumer acceptability of koji fish sauces and traditional fish sauces, fish sauce made with *Aspergillus oryzae* OAY1 had a similar acceptance rate (Sun et al., 2016).

Yeast starter cultures, used in many fermented products as starter cultures, can also control biogenic amines. *Omer kodak* yeast M8 can degrade up to 75% of biogenic amines under fish sauce fermentation conditions. This yeast starter culture also showed enhanced desired flavors and decreased rancid smells (Wang et al., 2014).

1.4. Food Safety

Bacterial pathogen risk is low in fish sauce due to two control measures inherently necessary for the preparation of the condiment – salt and fermentation. The lowering of water activity by salting, such as in the salting of anchovies to be fermented, creates an environment in which pathogenic bacteria are not able to effectively repopulate (Verdos et al., 2019). The microbial population responsible for the fermentation in the fish sauce is able to prevent pathogen growth due to the competition for nutrients or competitive exclusion (Hibbing et al., 2010). Additional health risks were identified through research in China which demonstrated a connection between fish sauce consumption and esophageal cancer. More studies are needed to establish a causal relationship (Ke et al., 2002). The primary food safety concern in fish sauce fermentation is the biogenic amine histamine production, which could cause histamine poisoning and cannot be controlled by a heat step.

1.4.1. Government Regulations

Due to the food safety concerns associated with histamine levels, the Food and Drug Administration in the United States created a standard for histamine allowed in fish products and specific species associated with histamine formation. They set a guidance level for histamine in edible portions of fish at 50 ppm (50 mg 100 mL^{-1}) (Food and Drug Administration, 2019). Since the product is exported worldwide, the United Nations set a non-legally binding standard. They set the upper limit of histamine in fish sauce at 40 mg histamine 100 g^{-1} (Food and Agriculture Organization of the United Nations & World Health Organization, 2011).

1.5. Conclusion and Experimental Objectives

Some of the current research regarding green crabs focuses on the control of the species through the establishment of a fishery. Although some studies have been conducted on making a value-added product, to our knowledge, none address the fermentation of this invasive species. Additionally, to our knowledge, there are no studies that address the control of histamine and the use of starter cultures in the fermentation of crab products. Despite the relatively low food safety risk associated with fish sauce, additional studies and data addressing the use of starter cultures could give background into how to control biogenic amines in other fermented products, particularly those with high protein content.

Therefore, the objectives of this research are (i) to determine the optimal salt concentration for fermentation of a green crab condiment, (ii) to determine the fermentation time needed to produce a fully fermented green crab condiment, (iii) to determine the optimal temperature for fermentation of a green crab condiment, (iv) to determine the effect of starter cultures (*Staphylococcus carnosus* and *Tetragenococcus halophilus*) on the concentration of histamine in a fermented green crab condiment, and (v) to assess the microbiota involved in the fermentation of a fermented green crab condiment.

1.6. References

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CHAPTER 2

USE OF INVASIVE GREEN CRAB *CARCINUS MAENAS* FOR PRODUCTION OF A FERMENTED CONDIMENT – AS PUBLISHED IN GREINER, D.M., SKONBERG, D.I., PERKINS, L.B., PERRY, J.J. (2021)

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2.1. Abstract

To control the population of an invasive species of green crab, we investigated the feasibility of producing a fermented crab condiment. Commercial fermented fish condiments were tested to assess variability in the marketplace and to identify targets for lab-fermented sauces. Finely chopped crab was combined with 100 mg g^{-1}, 200 mg g^{-1}, or 300 mg g^{-1} NaCl, and spontaneously fermented for up to 120 days. Chromatographic analysis revealed that histamine content was not a safety concern as all treatments were below the current U.S. legal threshold (50 mg 100 mL^{-1}). The majority of microbial and physicochemical properties measured within salt level (proteolytic bacterial population, total volatile basic nitrogen (TVBN), amine nitrogen, water activity, moisture, and biogenic amines) were statistically unchanged between days 60 and 120 of fermentation, suggesting that most of the biochemical changes happened early in the fermentation. While the production of a fermented condiment was successful and could represent an opportunity for the valorization of this invasive species, additional work is needed to accelerate the process and further understand the dynamics of the early fermentation stages.

2.2. Introduction

The European green crab (*Carcinus maenas*) is an extremely aggressive invasive species established on North America's east and west coasts (Lovell et al., 2007; St-Hilaire et al., 2016). The green crab preys on commercially relevant clam and mussel species, which has adversely affected coastal regions ecologically and economically. This predation has decreased soft-shelled clam populations on the east coast of the United States by 40%, representing a loss of $22.6 million per year in sales (Lovell et al., 2007). Adverse effects of this invasive species are not limited to predation. Mature and juvenile green crabs damage important ecosystems by eating eelgrass and digging through the sediment surrounding eelgrass (Malyshev & Quijón, 2011). These eelgrass beds are a critical habitat, serving as nurseries for juvenile fish, providing protection from native predators, and acting as an essential food resource for diverse species including waterfowl (Heck et al., 2003). The presence of green crabs also poses a threat to the American lobster population through competition over prey (Williams et al., 2006).

Unfortunately, hard shelled green crabs are difficult to use in the culinary industry because of difficulties removing meat from this diminutive species. One opportunity to control this species that has been investigated is the establishment of a soft-shell fishery. This fishery could help exert local control over the population of green crab and is considered low barrier to entry due to the use of existing fishing materials and the lack of trapping limits on this species (St-Hilaire et al., 2016). In Italy, there are mature soft-shell green crabs selling for as much as €51.14 kg^{-1}, or about $27 USD lb^{-1} (St-Hilaire et al., 2016). Other current uses for the crabs include composting and bait (Department of Marine Resources, 2014), but the establishment of a soft-shelled industry represents the highest value opportunity for fishers.

Pre-molt green crabs are easily identified and can be stored live until suitable for sale in a soft-shell market. However, a significant portion of the trapped crabs are not suitable for live storage. Although this excess biomass could be sold as bait or composted, the establishment of an edible use for the excess material would command a higher price for fishers. This additional valorization of excess biomass further incentivizes fishers and makes the system more sustainable (Teigiserova et al., 2020).

Fermented fish sauce is a clear liquid, brown in color, with a distinct fish flavor (Lopetcharat et al., 2001), which, in its simplest form, consists of the liquid resulting from the spontaneous fermentation of salted whole anchovies and other fish species (Martínez-Álvarez, O, López-Cabellero, M.E., Gómez-Guillén, M.C., Montero, 1967). The fermentation of a fish sauce typically ranges between 6 and 18 months (El Sheikha & Montet, 2014; Lopetcharat et al., 2001), though duration depends on ambient temperature and local preferences. Fermented seafood sauce is historically a widely used condiment in a large number of cultures, particularly those with Asian influence, and is becoming more common in American kitchens (America's Test Kitchen, 2016).

Anchovies are commonly used in fish sauce manufacture because of their low value and abundance of protein, with the Peruvian anchovy having up to 20% protein (Pariona-Velarde et al., 2020). Although anchovies are the most commonly used substrate, diverse species including tilapia, sardines, silver barb fish, and freshwater crab have been used in fermented condiment production (Achi et al., 2007; Brillantes et al., 2002; Kopermsub & Yunchalard, 2010; Lee et al., 2016; Saithong et al., 2010). *Carcinus maenas* has a protein content of about 17% (Skonberg & Perkins, 2002), which could be fermented as a way to utilize this valuable protein in the culinary industry without the need to separate the shell and meat. Additionally, this process is relatively low input with little capital investment needed, making this product accessible for small-scale processing operations or cottage producers. The purpose of this work was to assess the physicochemical properties of existing commercial fish sauce products to establish baseline values for a novel, fermented seafood condiment, and to assess the feasibility of using whole green crab as the substrate for such a product.

2.3. Materials and Methods

2.3.1. Preparation of Crab

Green crabs were trapped off the coast of Georgetown, Maine, and transported on ice to the University of Maine (Orono, ME, USA). Live crabs were blast frozen (Southeast Cooler, Lithia Springs, CA, USA) for 1 h at −30 °C, then stored at −20 °C until use. Frozen whole crabs were thawed for 36–48 h at 4 °C before being finely chopped in a Kolsch bowl cutter (UltraSource, Kansas City, MO, USA) and combined with uniodized Kosher salt (Morton Salt, Chicago, IL, USA) at 100 mg g^{-1}, 200 mg g^{-1}, or 300 mg g^{-1} (*w*/*w*). All treatments were prepared separately, in triplicate, and packed into clean, 0.95 L canning jars covered with a double layer of cheesecloth (Pyrm Consumer USA, Spartanburg, SC, USA).

2.3.2. Fermentation and Sampling

Treatments were incubated at 24 °C for 120 days with intermittent sampling occurring at 60, 90, and 120 days. On each sampling occasion one entire jar from each treatment replicate was utilized (no repeated sampling). The sauce was separated from the solid residue for testing by straining through two layers of non-sterile cheesecloth into 250 mL centrifuge tubes. The filtrate was centrifuged in an Avanti J-E Beckman Coulter centrifuge (Brea, CA, USA) (100× *g*, 10 min), and the supernatant was collected for microbial and physicochemical testing.

2.3.3. Determination of Microbial Activity

Crab sauce was serially diluted in 1 g L^{-1} peptone (Becton, Dickinson, and Co., Sparks, MD, USA) and spread plated in duplicate on skim milk agar (SMA), which consisted of brain heart infusion agar (Hardy Diagnostics, Santa Maria, CA, USA). It was supplemented with 100 mL L^{-1} (*v*/*v*) aseptically packaged skim milk (Natrel, Quebec, Canada) and 30 mg g^{-1} salt (Aqua Solutions, Deer Park, TX, USA; incubated at 37 °C for 48 h) to identify proteolytic bacteria (Zaman et al., 2011). The brain heart infusion agar was supplemented with 30 mg g^{-1} salt (incubated at 37 °C for 48 h) for total plate count (TPC), and potato dextrose agar (APDA; Alpha Biosciences, Baltimore, MD, USA) was acidified with 0.1 M tartaric acid (Alfa Aesar, Ward Hill, MA, USA; incubated at ambient temperature for 5 day) to isolate fungi. All plates were counted with colony density between 30 and 300, and the microbial population was expressed as log CFU g^{-1}.

2.3.4. Determination of Total Volatile Basic Nitrogen (TVBN) and Amine Nitrogen

Total volatile basic nitrogen was measured (Botta et al., 1984) via direct distillation with sodium hydroxide. Lab fermented crab sauce sample was homogenized with trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (1312× *g* 20 min). The supernatant was distilled in a micro-Kjeldahl apparatus (Labconco, Kansas City, MO, USA) with sodium hydroxide (Fisher Chemical, Fair Lawn, NJ, USA) and antifoaming agent A (Sigma Aldrich, St. Louis, MO, USA). The distillate was collected in boric acid solution (JT Baker, Center Valley, PA, USA) containing methyl-red (Fisher Scientific, Waltham, MA, USA), a methylene- blue (Sigma Aldrich, St. Louis, MO, USA) indicator, and titrated for a color change with 0.1 N hydrochloric acid (Fisher Scientific, Waltham, MA, USA). TVBN content was expressed as mg 100 mL^{-1}.

Proteolytic activity drives increased amine nitrogen content (Zhou et al., 2016). This measurement is typically used to determine the degree of proteolysis due to activity of endogenous and microbial proteases and can be used to estimate the progress of fermentation. The amine nitrogen was determined using a formol titration published by (Joung & Min, 2018), with the following slight modifications. A concentration of 0.1 N sodium hydroxide (NaOH) was used for the neutralization of the original sample and formaldehyde. Moreover, 8 mL of formaldehyde was added (Fisher Scientific, Waltham, MA, USA). The titrant used for the mixture of neutralized formaldehyde and neutralized crab sauce sample was 0.05 N NaOH. Data were expressed as mgN 100 mL^{-1}.

2.3.5. Determination of pH, Water Activity, and Moisture Content

The pH (Orion Star A111 pH meter, Thermo Scientific, Waltham, MA, USA) was determined through a single, direct reading from each sample. The probe was first calibrated with pH 4, 7, and 10 standards. The water activity values (Aqualab, Pullman, WA, USA) were determined through two direct readings and averaged. The water activity meter was first calibrated with a 0.76 calibration standard. Moisture content (%) was determined using AOAC Method 934.01 (AOAC Official Method 934.01, 2005) at a vacuum of 20 in Hg.

2.3.6. Determination of Non-Enzymatic Browning

Non-enzymatic browning was measured according to the method of (Zhao et al., 2017) with slight modification. One mL of sauce was stirred using a magnetic stir bar and plate with 10 mL of ethanol (500 mL L^{-1} *v/v* Fisher Scientific, Waltham, MA, USA) for one hour. The mixture was then filtered through a 0.45 µm syringe filter (MDI Membrane, Harrisburg, PA, USA) and subjected to an absorbance measurement at 420 nm with a DU 530 spectrophotometer (Beckman Coulter, Brea, CA, USA).

2.3.7. Determination of Biogenic Amine Content

Biogenic amines in samples and analytical standards were determined using high performance liquid chromatography (HPLC) and the Waters AccQ-Fluor^{TM} fluorescent tagging system (Milford, MA, USA), a method developed for the determination of amine compounds in foods. The Agilent model 1100/1200 HPLC system included a quaternary pump, autosampler, column heater, fluorescence detector, and ChemstationTM software. Approximately 1 mL of crap sauce was filtered through a 0.45 µM nylon syringe filter (Cole-Palmer, Vernon Hills, IL, USA). Ten µL of sample filtrates and 10 µL of standards were prepared for HPLC analysis with the AccQ·Fluor^{TM} kit and assayed using the HPLC column and eluents supplied with the kit. All procedures included in the kit directions insert were followed, with a slight modification of the HPLC gradient elution. Standard curves were constructed using five concentrations of histamine, agmatine, putrescine, cadaverine, and tyramine (all from Sigma-Aldrich, St. Louis, MO, USA), ranging from 0.557–0.894 mg mL^{-1} and diluted with HPLC-grade water. Baseline separation of the target analytes was achieved and biogenic amines were identified by comparing retention times from samples with the analytical standards. Peak areas were used to calculate analyte concentrations. Data were expressed as mg 100 mL^{-1}.

2.3.8. Commercial Fish Sauces

In addition to laboratory-fermented crab sauce, 12 varieties of commercial fish sauce were obtained from local and online retail outlets. These sauces were subjected to the same analyses described above to create a standard for comparison since fermented crab sauce is not commercially available. Commercial sauces were separated into two tiers (Table 1) according to ingredients (Tier 1 comprised of minimal ingredients suggesting traditionally fermented, higher value product, Tier 2 comprised of sauces containing additional ingredients such as colorants and preservatives) and were compared to each other statistically to identify significant differences. Tier 1 samples, on the basis of higher quality, were identified as better targets for laboratory-fermented sauce and were subsequently compared statistically to day 120 prototypes.

2.3.9. Statistical Analysis

The data were tested for normality using a Shapiro-Wilks test. Outliers were identified and removed when appropriate. The results were analyzed for variance using multivariate ANOVA for normal data and Kruskal–Wallis test for non-normal data. Tukey's honestly significant difference (HSD) post hoc test was used to identify statistically significant ($p < 0.05$) differences among treatments after testing for variance in R Version 3.6.1 (R Studio, Boston, MA, USA).

2.4. Results

2.4.1. Commercial Fish Sauces

In comparing the ingredients of commercial sauces currently available in the marketplace, two distinct groups emerged. One of these (Tier 1) contained only minimal ingredients characteristic of a traditionally fermented fish sauce, while a second (Tier 2) contained various additional, "non-traditional" ingredients. In order to allow for the calculation of mean values for comparison to lab-fermented sauce, the commercial sauces were separated into two categories (Table 2.1.).

Between the two tiers of commercial product, there were apparent differences in water activity, TVBN, and amine nitrogen. These differences were statistically significant and suggest that the use of traditional vs. non-traditional ingredients results in a different sauce. Tier 2 sauces had a significantly higher water activity, and significantly lower TVBN and amine nitrogen contents than tier 1 sauces.

The commercial sauces made from traditional ingredients—i.e., fish, salt, and some- times sugar and water—were selected as a more appropriate target for the experimental product. They were compared to the lab-fermented crab sauces to identify properties of significant difference. The lab fermented crab samples had a significantly higher pH at all salt levels. The water activity of the 100 mg g^{-1} salt lab-fermented sample (0.857 ± 0.004) was also significantly higher than the mean of the tier 1 commercial samples (0.74 ± 0.01), but no difference between higher salt and commercial formulations was detected.

Table 2.1. Commercial Fish Sauce Physicochemical Characteristics

	Tier 1[a] (n=5)	**Tier 2[b] (n=7)**
Ingredients[c]	anchovy fish, sea salt, sugar, water	anchovy extract, water, salt, mackerel extract, potassium sorbate, fermented scad fish extract, caramel color, syrup, sugar
pH	5.56 ± 0.29	5.41 ± 0.58
Water Activity	0.739 ± 0.014 *	0.774 ± 0.027 *
TVBN (mgN $100mL^{-1}$)	390.5 ± 129.4 *	138.3 ± 141.9 *
AmineN (mgN $100mL^{-1}$)	1271.2 ± 528.8 *	449.0 ± 447.8 *
Moisture (%)	59.3 ± 4.1	65.9 ± 6.8
Browning	0.53 ± 0.24	0.29 ± 0.29
Histamine (mg $100mL^{-1}$)	3.49 ± 1.69	1.82 ± 0.61
Putrescine (mg $100mL^{-1}$)	3.93 ± 2.84	3.50 ± 3.62
Cadaverine (mg $100mL^{-1}$)	0.81 ± 0.24	0.75 ± 0.42
Tyramine (mg $100mL^{-1}$)	2.09 ± 0.41	1.46 ± 0.81

Data are expressed as mean ± standard deviation
* indicates significant differences in mean values between tiers
[a]Includes the following brands: A Taste of Thai, Four Elephants, Son Sauce, Red Boat, Golden Boy
[b]Includes the following brands: Nuoc Mam Nhi, Purfina Patis, Tentay Patis, Lucky, Mega Chef, Essential, Squid Brand
[c]Individual sauce ingredients comprise various combinations of listed ingredients within tier

2.4.2. Microbial Activity

Yeast and mold levels in general (Table 2.2.) were low in all treatments and did not have any significant differences due to treatment or time during the fermentation.

Table 2.2. Fungal Population in Lab Fermented Crab Sauces

Time (Day)	100mg g^{-1} Salt ($logCFUg^{-1}$)	200mg g^{-1} Salt ($logCFUg^{-1}$)	300mg g^{-1} Salt ($logCFUg^{-1}$)
60	2.5 ± 0.7	2.7 ± 0.5	2.5 ± 0.8
90	2.1 ± 0.1	2.0 ± 0.0	2.0 ± 0.0
120	2.2 ± 0.1	2.2 ± 0.2	2.5 ± 0.4

Data are expressed as mean ± standard deviation (n = 3)

No distinct trends were found in the levels of any of the microorganisms of interest (total count, proteolytic bacteria, fungi). Based on the model level effects, an increase in salt content negatively affected the proteolytic bacteria (Figure 2.1.) population. However, the only significant difference among treatments was between 100 mg g^{-1} and 300 mg g^{-1} salt samples (6.6 ± 0.3 logCFU g^{-1} and 3.5 ± 0.4 logCFU g^{-1} respectively) on day 120.

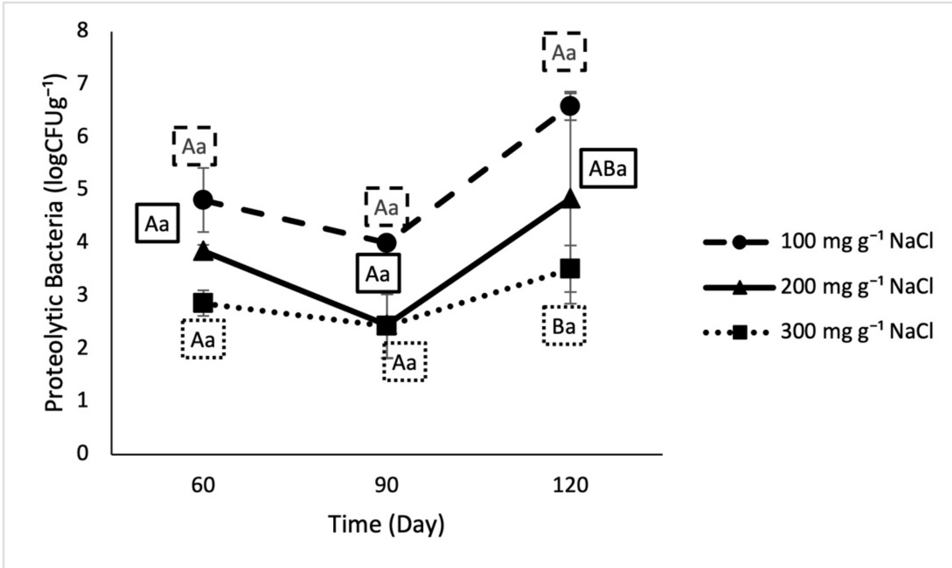

Figure 2.1. Proteolytic Bacterial Population in Lab Fermented Crab Sauce Samples Over Time

Uppercase letters designate significant differences among treatments on individual testing dates. Lowercase letters designate significant differences within treatment across time. (n=3)

Both longer fermentation time and higher salt content had significant model level effects on TPC (Figure 2.2.) that resulted in increased plate count.

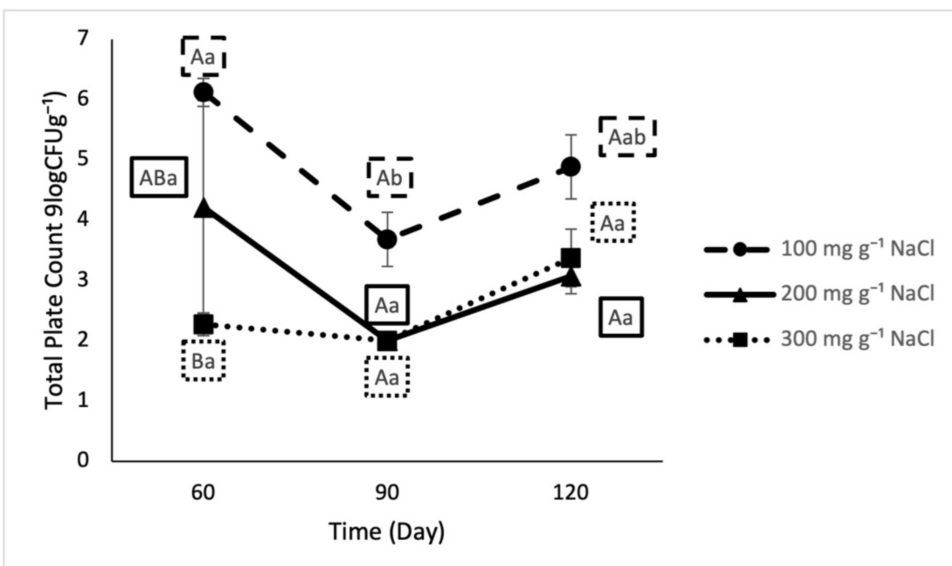

Figure 2.2. Total Plate Count in Lab Fermented Crab Sauce Samples Over Time

Uppercase letters designate significant differences among treatments on individual testing dates. Lowercase letters designate significant differences within treatment across time. (n=3)

2.4.3. TVBN and Amine N

In our analysis, TVBN did not increase over time, but crab sauce formulated with 100 mg g^{-1} salt had significantly higher levels of TVBN (287.9 ± 14.0 mg 100 mL^{-1}) than the 200 mg g^{-1} and 300 mg g^{-1} samples (138.2 ± 17.6 and 134.8 ± 11.7 mg 100 mL^{-1} respectively) at all time points (Figure 2.3.). The 200 mg g^{-1} and 300 mg g^{-1} salt treatments were statistically indistinguishable from each other regardless of time.

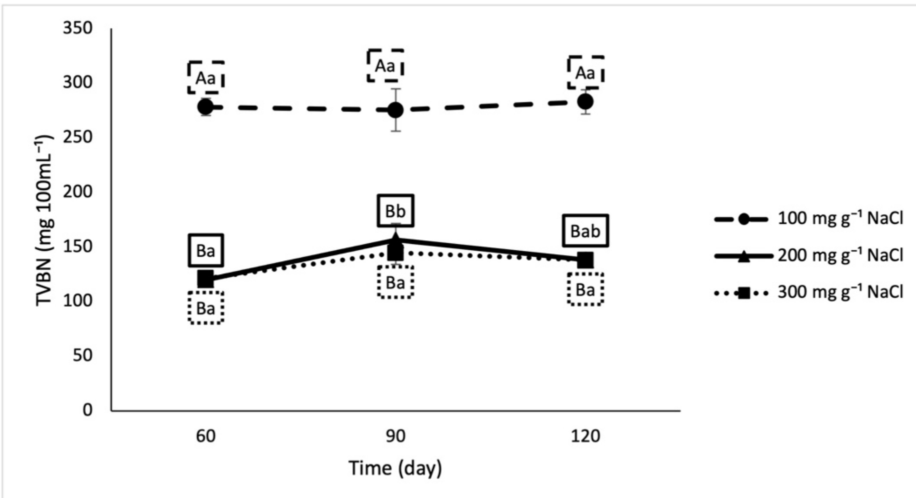

Figure 2.3. Total Volatile Basic Nitrogen in Lab Fermented Crab Sauce Samples Over Time

Uppercase letters designate significant differences between treatments on individual testing dates. Lowercase letters designate significant differences within treatment across time. (n=3)

Although there were significant differences in amine nitrogen levels among treatments, with the 100 mg g^{-1} salt treatment having significantly higher amine nitrogen (907.67±47.72 mg N 100 mL^{-1}) than the 200 mg g^{-1} and 300 mg g^{-1} samples (628.06 ± 33.44 and 581.00±39.92 mgN 100 mL^{-1} respectively), the amine nitrogen levels did not increase over time in any treatment (Table 2.3.).

Table 2.3. Amine Nitrogen in Lab Fermented Crab Sauce Samples

Time (Day)	100 mg g^{-1} Salt (mgN 100 mL^{-1})	200 mg g^{-1} Salt (mgN 100 mL^{-1})	300 mg g^{-1} Salt (mgN 100 mL^{-1})
60	884.3 ± 56.5 $_a$	581.0 ± 17.4 $_b$	539.0 ± 9.9 $_b$
90	864.5 ± 168.5 $_a$	655.7 ± 1.6 $_b$	569.33 ± 46.2 $_b$
120	974.2 ± 24.6 $_a$	647.5 ± 32.2 $_b$	634.7 ± 10.0 $_b$

Lowercase letters designate significant differences between treatments on individual testing dates. Data are expressed as mean ± standard deviation (n = 3).

2.4.4. pH, Water Activity, and Moisture Content

Differences in pH were driven by time (Figure 2.4.). From day 90 to 120, the pH of the higher salt samples did not change, but the mean pH of the 100 mg g^{-1} salt sample significantly decreased.

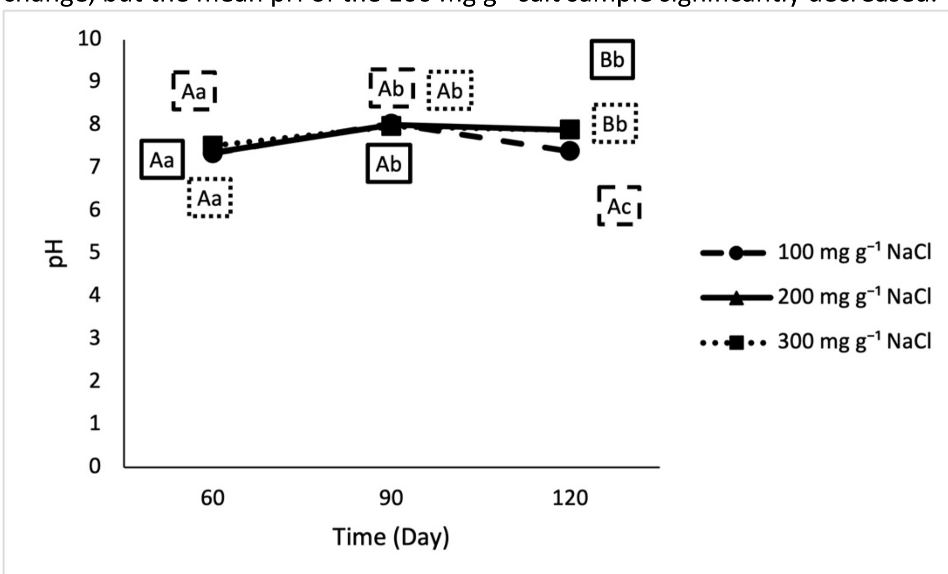

Figure 2.4. pH in Lab Fermented Crab Sauce Samples Over Time

Uppercase letters designate significant differences between treatments on individual testing dates. Lowercase letters designate significant differences within treatment across time. (n=3)

Crab sauces made with 200 mg g^{-1} (0.748 ± 0.005) and 300 mg g^{-1} (0.744 ± 0.004) salt had significantly ($p<0.05$) lower water activities than those prepared with 100 mg g^{-1} salt (0.861 ± 0.006) at all time points (Table 2.4.), as expected.

Table 2.4. Water Activity in Lab Fermented Crab Sauce Samples

Time (Day)	100mg g^{-1} Salt	200mg g^{-1} Salt	300mg g^{-1} Salt
60	0.866 ± 0.004 $_a$	0.744 ± 0.001 $_b$	0.741 ± 0.001 $_b$
90	0.862 ± 0.005 $_a$	0.751 ± 0.007 $_b$	0.744 ± 0.003 $_b$
120	0.857 ± 0.004 $_a$	0.749 ± 0.002 $_b$	0.748 ± 0.002 $_b$

Lowercase letters designate significant differences between treatments on individual testing dates. Data are expressed as mean ± standard deviation (n = 3).

Similar to water activity, the moisture content (Table 2.5.) of samples made with 200 mg g^{-1} (66.61 ± 2.13%) and 300 mg g^{-1} of salt (68.32 ± 0.43) was significantly lower than those prepared with 100 mg g^{-1} salt (74.52 ± 0.95%) at all time points.

Table 2.5. Moisture in Lab Fermented Crab Sauce Samples

Time (Day)	100mg g^{-1} Salt (%)	200mg g^{-1} Salt (%)	300mg g^{-1} Salt (%)
60	75.8 ± 0.4 $_a$	67.5 ± 0.3 $_b$	68.6 ± 0.6 $_b$
90	73.8 ± 2.8 $_a$	68.7 ± 0.7 $_b$	68.7 ± 2.8 $_b$
120	73.9 ± 1.9 $_a$	63.7 ± 9.0 $_b$	67.7 ± 0.4 $_b$

Lowercase letters designate significant differences between treatments on individual testing dates. Data are expressed as mean ± standard deviation (n = 3).

2.4.5. Non-Enzymatic Browning

Non-enzymatic browning is the development of the brown color of the sauce caused by reactions such as the Maillard reaction. The Maillard reaction primarily involves the reaction of reducing sugars, oxidation products, and free amino acids (Lopetcharat et al., 2001). The degree of non-enzymatic browning (Figure 2.5.) of samples fermented with 200 mg g^{-1} and 300 mg g^{-1} salt was significantly higher than that observed in the 100 mg g^{-1} samples on days 60 and 90, but results were not significantly different due to treatment on day 120. The non-enzymatic browning on day 120 (0.237 ± 0.00) was found not to be significantly different from the Tier 1 commercial samples at any of the salt levels.

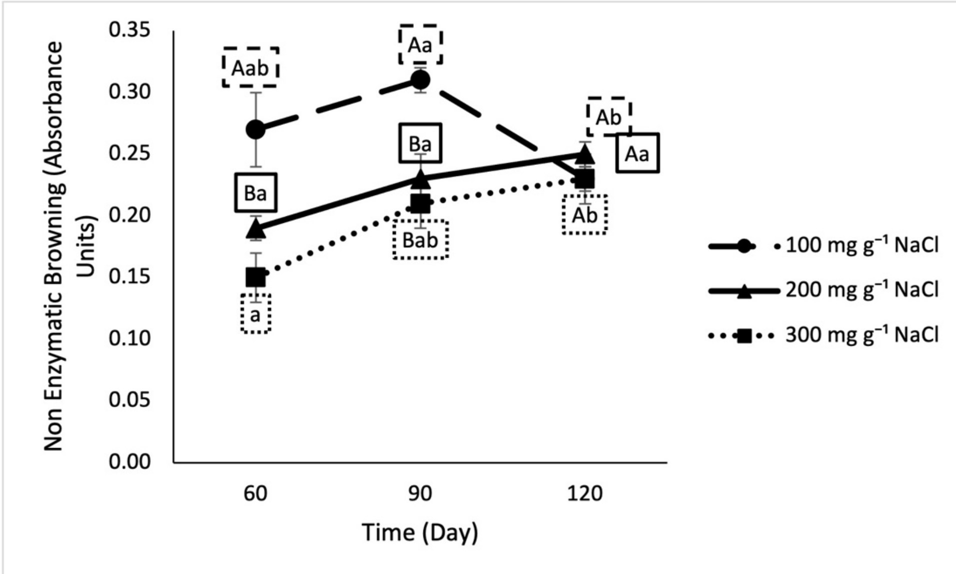

Figure 2.5. Non-Enzymatic Browning in Lab Fermented Crab Sauce Samples Over Time

Uppercase letters designate significant differences between treatments on individual testing dates. Lowercase letters designate significant differences within treatment across time. (n=3)

2.4.6. Biogenic Amines

There was no significant effect on the biogenic amine concentration (Table 2.7.) from time or salinity. Data are expressed as averages among time and salinity. Cadaverine and tyramine were not identified in any of the crab sauces.

Table 2.6. Biogenic Amine Concentration of Lab Fermented Crab Sauce Samples

Biogenic Amine	Average Concentration (mg 100 mL^{-1})
Histamine	6.01 ± 1.54
Putrescine	3.50 ± 1.69
Agmatine	4.34 ± 5.07

Data are expressed as mean ± standard deviation (n = 3)

2.5. Discussion

Fish sauce is traditionally made through spontaneous fermentation. During spontaneous fish sauce fermentation, proteolytic enzymes break down fish flesh. This proteolysis is induced by endogenous proteases found in the muscles and the digestive tracts of the fish/crab and the activity of natural microbiota. The speed at which this degradation occurs depends on a number of factors including the amount of salt in the formulation and the incubation temperature of the raw materials. The catabolism of proteins yields a varied assortment of nitrogenous compounds responsible for the aroma and flavor of traditional fish sauce (Zheng et al., 2017). This spontaneous fermentation was the method used to make a fermented condiment from *Carcinus maenas* in this study. The formulations studied consisted of crab and 100 mg g^{-1}, 200 mg g^{-1}, or 300 mg g^{-1} salt.

In traditional fish sauce fermentation, proteolytic bacteria are expected to be present at populations of almost 4.0 logCFUg^{-1} after 4 months (Yuen et al., 2009; Zaman et al., 2011). The proteolytic bacterial population in this study ranged from 2.4-3.5 logCFU g^{-1} after 120 days of fermentation. As the fermentation progresses, the enzymes produced by these bacteria catabolize the proteins in the raw material, resulting in the formation of peptides and amino acids, measured in this study as amine nitrogen. Total volatile base nitrogen also increases with time, giving rise to odiferous compounds that drive the use of this metric as an indicator of spoilage in fish (Pacquit et al., 2006). Because they result from the breakdown of proteins, which characterizes the process of fish sauce fermentation, both of these indices could be expected to increase with increasing fermentation duration (to almost 2400 mg 100 mL^{-1} and 600 mg 100 mL^{-1} respectively) (Y. Wang et al., 2018; Zaman et al., 2011). That was not, however, observed in our study. Data in this study suggests that the majority of protein catabolism occurred in the very early stages of the fermentation.

In some instances, depending on the microbiota present in the system, amino acids are decarboxylated to release biogenic amines, such as histamine. Biogenic amine content can be an indicator of product quality and aids in determining acceptability of a commercial sample. A high histamine content specifically can cause scombroid poisoning, which causes rash, swelling, and vomiting

(Wang et al., 2018). As a result, in the United States, fish sauce cannot legally contain more than 50 mg 100 mL^{-1} of histamine (Food and Drug Administration, 2019). Histamine levels were low in experimental product, which could suggest that the native flora of *Carcinus maenas* is largely non-histamine producing. Agmatine was not recovered from most commercial samples but was frequently identified in lab fermented sauces, likely due to the difference of arginine content in the starting material. None of the lab fermented samples or commercial samples had a histamine concentration above the legal limit.

Water in foods drives a number of degradative processes. Water activity measures the amount of free water available for bacterial growth (Brady, 2013). Most pathogenic bacteria are unable to grow at a water activity below 0.9 (Fellows, 2000), indicating that vegetative pathogens are unlikely to pose significant food safety concerns for this fermented crab product, particularly in the higher salt formulations, which both had water activity values well below 0.8.

There is very little information in the literature regarding the sensory aspects driving acceptability of fermented fish sauces. As a result of this fact, we chose to complete a small scale survey of commercial products in order to orient the experimental crab sauce in comparison to potential physicochemical targets. The resulting fermented crab condiment looked and smelled similar to the commercial fish sauces in that it was a dark brown color and smelled like fermented seafood. The pH was higher in all lab samples than the commercially available samples, most likely due to the higher levels of lactic acid bacterial population associated with fish sauce fermentation (Saithong et al., 2010). There were no differences between the lab and commercial samples in amine nitrogen content, which shows that the degree of proteolysis that occurred in the lab samples was comparable to the commercial samples. Comparable levels of browning suggest that the color of the experimental crab sauce has the potential to meet current consumer expectations. Levels of TVBN and moisture (in all salt formulas) were also similar to commercial product. However, better characterization of the first 60 days of fermentation is warranted. Likewise, consumer acceptability testing is needed in order to assure the palatability of a green crab sauce. Additional investigation should also assess whether the process could be accelerated or consumer appeal could be increased by modification of formula or process alterations.

2.6. Conclusions

The concept of creating a fermented condiment from *Carcinus maenas* has proven to be a feasible opportunity. The experimental crab sauce product was comparable in many respects to currently available commercial fish sauce products. Optimization of the product and process, as well as consumer sensory evaluation, are called for to ensure the market potential of such a product. The establishment of an economically feasible use for green crabs unsuitable for the high value softshell market will be a key accomplishment in facilitating the development of a viable US fishery for this invasive species and realizing the associated ecological benefits of controlling its population.

2.7. Author Contributions

Conceptualization, D.S. and J.P.; methodology, all authors; formal analysis, D.G.; data curation, all authors; writing—original draft preparation, D.G.; writing—review and editing, J.P., D.S. and L.P.; visualization, D.G.; supervision, D.S. and J.P.; funding acquisition, D.S. and J.P. All authors have read and agreed to the published version of the manuscript.

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2.9. Data Availability Statement

Data available upon request from corresponding author.

2.10. Acknowledgements

We would like to acknowledge Rosanna Woodruff for processing the crabs and conducting laboratory analyses as well as Marissa McMahan for providing the crabs.

2.11. Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish results.

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CHAPTER 3

THE OPTIMIZATION OF *CARCINUS MAENAS* FERMENTATION BY TEMPERATURE CONTROL

3.1. Abstract

To optimize the fermentation of *Carcinus maenas*, various temperatures of fermentation were investigated. Ground crab was combined with 200 mg g^{-1} NaCl and spontaneously fermented for up to 90 days at 24°C, 30°C, 37°C, or 50°C. Chromatographic analysis revealed the histamine concentration was substantially below legally allowed limits of 50 mg 100 mL^{-1}. Microbial enumeration indicated that peak population levels were reached at 15 days of fermentation with declines in all populations (proteolytic, histamine forming, and lactic acid bacteria) observed for the rest of the fermentation. Chemical testing (water activity, amine nitrogen, and non-enzymatic browning) indicated a recommended temperature for the fermentation of a crab sauce is between 30°C and 37°C, but a fermentation temperature of 24°C or 50°C may also result in production of an acceptable sauce. While this investigation of temperature offered an idea of the most chemically favored fermented green crab sauce, further research is needed to confirm consumer acceptability of this condiment.

3.2. Introduction

The invasive species of green crab *Carcinus maenas* was introduced to the East Coast of the United States in the early 1800s (Klassen & Locke, 2007). Since their initial invasion, these green crabs have been the cause of both ecological and economic problems within the Gulf of Maine. Ecologically, the crabs are found competing with American lobsters (Williams et al., 2006) for food as well as digging in and uprooting eelgrass beds (Malyshev & Quijón, 2011). The crabs also prey heavily on bivalves (Congleton et al., 2006), not only affecting the ecosystem but also hurting the soft shell clam industry in the Gulf of Maine. Due to this predation, between the years 1975 and 2005, the clam industry on the east coast has lost an estimated US $700 million (Lovell et al., 2007).

Although the green crabs have been present on the North American East Coast for about 200 years, the population density of the green crabs has become more problematic for humans in the last 30-50 years (Klassen & Locke, 2007). Green crab predation effects have become more detrimental to the ecosystem with warming climate temperatures. Due to the increased winter ocean surface temperature, the amount of winterkill in green crabs has decreased, allowing for more predation opportunities (Congleton et al., 2006).

A potential solution for this uncontrolled population of invasive green crabs is the establishment of a fishery. In Italy, soft shell green crabs can sell for as much as $27 lb^{-1} (Lovell et al., 2007). The establishment of a green crab fishery in the United States is technologically feasible as readily accessible existing materials can be utilized (McMahan & Bradt, 2020), but there is a large accumulation of crab biomass that cannot be sold for a high value. Currently on the North American East Coast, soft-shell green crabs can be sold in limited quantities for up to $25 lb^{-1} directly to restaurants (McMahan, 2021), but excess biomass from green crab fishing can only be sold as compost (Department of Marine Resources, 2014) or lobster bait (Klassen & Locke, 2007), both low value waste streams. The crabs are too small to easily be picked, so a culinary hard-shell use is not feasible. There is a need to provide a higher value waste stream to ensure financial sustainability of green crab fisheries.

The feasibility of a fermented green crab condiment has been explored and validated through previous research (Greiner et al., 2021). However, for commercial production, a deeper understanding of the factors contributing to product and process optimization is called for. A spontaneous fermentation process can be optimized by the adjustment of formula (salt concentration) and the adjustment of temperature, as well as the addition of starter cultures and/or enzymes. The purpose of this work was to investigate the impact of variations in fermentation temperature on the progress of the fermentation and physicochemical characteristics of the resulting fermentate.

3.3. Materials and Methods

3.3.1. Preparation of Crab

Green crabs were caught off the coast of Georgetown, Maine and transported on ice to the University of Maine (Orono, ME). Live crabs were frozen until dead at -20°C and stored at -20°C for two months prior to use. The whole, frozen crabs were thawed for 36-48 hours at 4°C. Crabs were finely ground in a meat grinder (Hobart Corporation, Troy, OH) using a 3/16 in die plate and combined with 200 mg g^{-1} (w/w) canning and pickling salt (Morton Salt, Chicago, IL). All crab was prepared for fermentation at four separate temperatures in triplicate in clean (nonsterile) 0.95 L glass canning jars containing approximately 800g of crab and salt mixture covered with a double layer of cheesecloth (Pyrm Consumer USA, Spartanburg, SC).

3.3.2. Fermentation and Sampling

The crab/salt mixture was incubated at 24°C, 30°C, 37°C, or 50°C for 90 days. After 15, 30, 60, and 90 days of fermentation one jar of each treatment replicate was analyzed (no repeated sampling). The sauce was separated from the solid particulate by filtering through two layers of cheesecloth into 250 mL centrifuge tubes, which were centrifuged in an Avanti J-E Beckman Coulter centrifuge (Brea, CA) at 150 x *g* for 10 min. The supernatant was collected for microbial and physiochemical testing.

3.3.3. pH and Water Activity

The pH of experimental crab sauce was determined through a single, direct reading from a Beckman 32pH meter (Brea, CA). The probe was first calibrated with pH 4 and 10 standards. The water activity values (Aqualab, Pullman, WA) were determined through two direct readings and averaged. The water activity meter was first calibrated with a 0.76 calibration standard (Aqualab, Pullman, WA).

3.3.4. Microbial Population

Crab sauce was serially diluted in 1 g L^{-1} peptone (Becton, Dickinson, and Co., Sparks, MD), spread plated (Zaman et al., 2011) in duplicate onto skim milk agar (SMA; Zaman et al., 2011) consisting of brain heart infusion agar (Hardy Diagnostics, Santa Maria, CA) supplemented with 100 mL L^{-1} (v/v) aseptically packaged skim milk (Natrel, Quebec, Canada) and 30 mg g^{-1} salt (Aqua Solutions, Deer Park, TX; incubated at 37°C for 48 h) to identify proteolytic bacteria, a synthetic differential medium for biogenic amine-producing bacteria supplemented with histidine (Zaman et al., 2011; incubated at 37°C for 48 h), and de Man, Rogosa, and Sharpe (MRS; BD Difco, Franklin Lakes, NJ; incubated at 30°C for 48 h) agar to identify lactic acid bacteria. All plates were counted with colony density between 30 and 300, and the microbial populations were expressed as log CFU g^{-1}.

3.3.5. Non-Enzymatic Browning

Non-enzymatic browning was measured using a modification of Zhao et al. (2017) as previously published in Greiner et al. (2021). One mL of sauce was stirred with 10 mL of ethanol (500 mL L^{-1} v/v; Fisher Scientific, Waltham, MA) for one hour, filtered through a 0.45 µm syringe filter (MDI Membrane, Harrisburg, PA) and subjected to an absorbance measurement at 420 nm with a DU 530 spectrophotometer (Beckman Coulter, Brea, CA). The spectrophotometer was blanked with 500 mL L^{-1} v/v ethanol between each sample. Data were measured in duplicate and expressed as Absorbance_{420}.

3.3.6. Amine Nitrogen

The amine nitrogen was determined using a modification of Joung & Min (2018) as previously published in Greiner et al. (2021). The sample was diluted (1mL of crab sauce with 9mL of water) and then neutralized to a pH of 8.5 with 0.1N NaOH. Eight mL of formaldehyde (37% v/v) neutralized to a pH of 8.5 was added. The mixture was titrated with 0.05N NaOH to pH 8.5. Data were expressed as mg N $100 mL^{-1}$.

3.3.7. Biogenic Amines

Biogenic amines in samples and analytical standards were determined using the method published by Greiner et al. (2021). About one mL of lab-fermented crab sauce was filtered through a 0.45 µM nylon syringe filter (Cole-Palmer, Vernon Hills, IL, USA) and 10 µL of both sample filtrates and standards were prepared for HPLC analysis using the AccQ·Fluor™ kit. Instructions in the kit were followed with a modification of elution gradient published by Greiner et al. (2021). Standard curves were constructed using concentrations of histamine, agmatine, putrescine, cadaverine, and tyramine (all from Sigma-Aldrich, St. Louis, MO) ranging from 0.49-1.33 mg mL^{-1} diluted with HPLC grade water. Peak areas identified by separation were used to calculate analyte concentrations. Data were expressed as mg 100 mL^{-1}.

3.3.8. Statistical Analysis

Data were analyzed using R version 4.0.3 (Vienna, Austria). Normality of the data was investigated using the Shapiro-Wilkes test. Normal data were tested for significant differences using a multiway analysis of variance (ANOVA). Non-normal data were tested for significant differences using a Kruskal-Wallis test. Tukey's Honestly Significant Difference (HSD) was used for post-hoc testing.

3.4. Results

3.4.1. pH and Water Activity

The interaction of fermentation temperature and time significantly affected the pH of the samples at the model level. The pH (Figure 3.1.) of the crab sauce fermented at 50°C increased significantly over time and yielded the highest final pH of any treatment. The pH of crab sauces fermented at all other temperatures (24°C, 30°C, and 37°C) decreased over time.

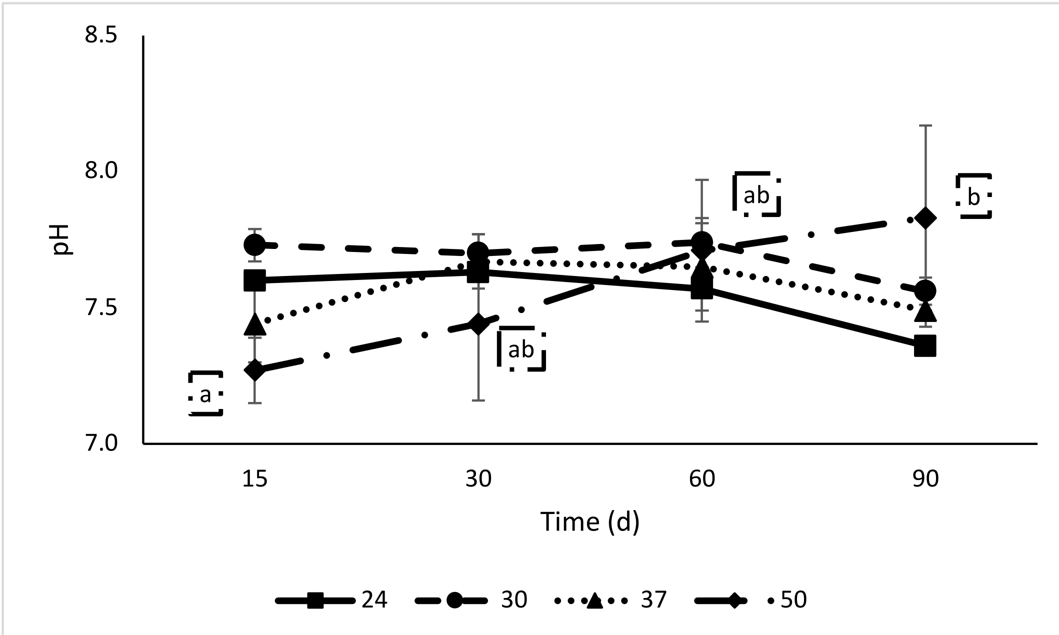

Figure 3.1. pH of Lab-Fermented Crab Sauces Over Time. Lowercase letters designate significant differences within treatment across time. The error bars represent the standard deviation. (n=3)

Fermentation temperature exerted significant effects on the water activity (Figure 3.2.). The crab sauce fermented at 24°C had a significantly higher water activity than the crab sauce fermented at 37°C throughout the fermentation after day 15. The crab sauce fermented at 24°C had a significantly higher water activity (0.765 ± 0.010) than crab sauces fermented at 30°C, 37°C, and 50°C (0.735 ± 0.006, 0.738 ± 0.007, and 0.730 ± 0.003 respectively) on day 60. The lowest water activity achieved in "finished" product (d 90) was observed in the 37°C treatment.

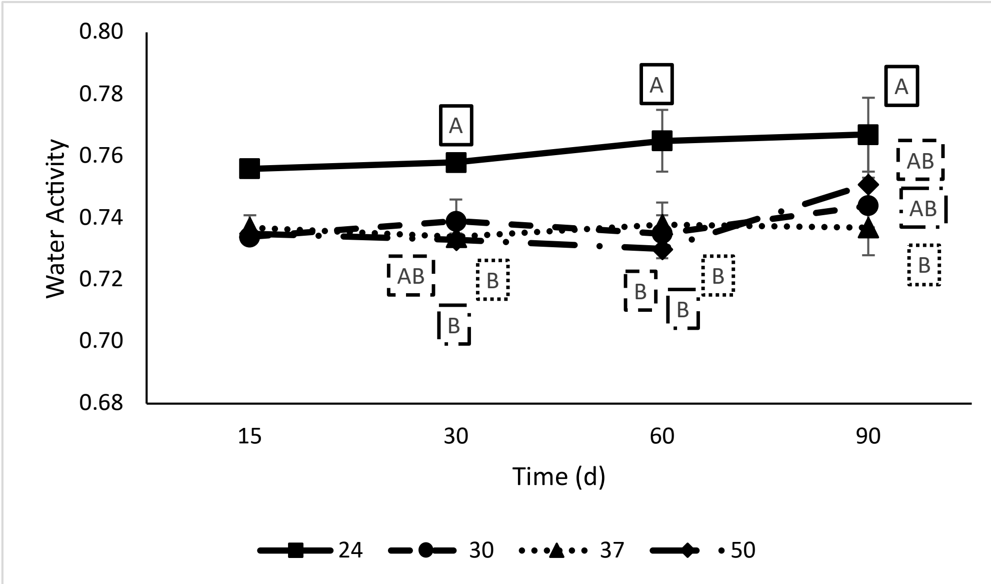

Figure 3.2. Water Activity of Lab-Fermented Crab Sauces Over Time. Uppercase letters designate significant differences between treatments on individual testing days. The error bars represent the standard deviation. (n=3)

3.4.2. Microbial Population

Fermentation temperature exerted significant effects on the proteolytic bacterial population. Population levels appeared to peak at 15 days, with sauce fermented at 37°C maintaining the lowest level. The proteolytic bacterial population (Figure 3.3.) in the crab sauces fermented at 24, 30 and 50°C was significantly higher on day 15 than day 90. The average proteolytic bacterial population at the end of the fermentation across all treatments was 2.3 ± 0.2 logCFU g^{-1}.

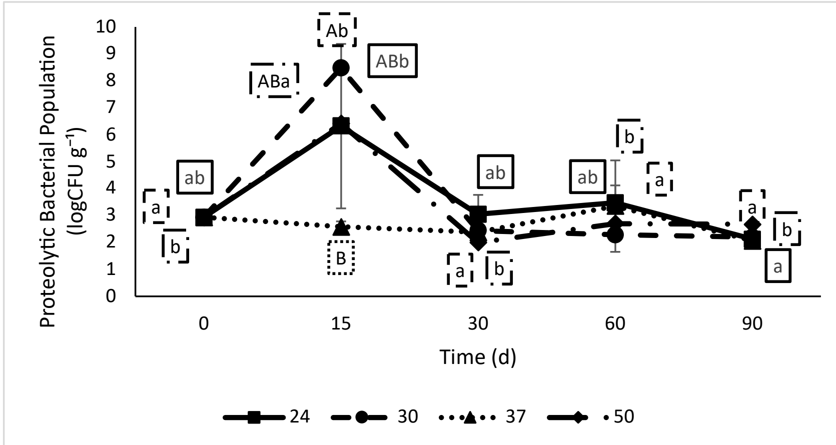

Figure 3.3. Proteolytic Bacterial Population of Lab-Fermented Crab Sauces Over Time. Uppercase letters designate significant differences between treatments on individual testing days. Lowercase letters designate significant differences within treatment across time. The error bars represent the standard deviation. (n=3)

The population of histamine forming bacteria (Figure 3.4) had a similar trend to the proteolytic bacteria, but was only significantly affected by fermentation time. The bacterial population in crab sauces fermented at 24°C, 30°C, and 50°C again peaked on day 15. The population of histamine forming bacteria in crab sauce fermented at 24°C (6.6 ± 2.7 logCFU g^{-1}) and 30°C (8.5 ± 0.0 logCFU g^{-1}) was significantly higher than in crab sauce fermented at 37°C (2.3 ± 0.0 logCFU g^{-1}) on day 15. On day 90 there were no significant differences due to fermentation temperature, yielding an average final population of 2.7 ± 0.6 logCFU g^{-1}.

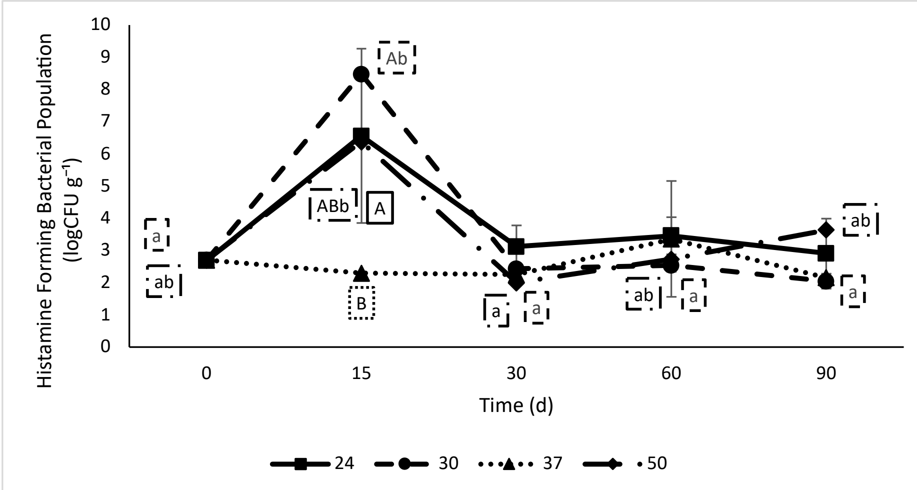

Figure 3.4. Histamine Forming Bacterial Population in Lab-Fermented Crab Sauces Over Time. Uppercase letters designate significant differences between treatments on individual testing days. Lowercase letters designate significant differences within treatment across time. The error bars represent the standard deviation. (n=3)

Fermentation time and temperature both exerted significant effects on lactic acid bacterial population. The lactic acid bacterial population (Figure 3.5.) also peaked on day 15 in crab sauces fermented at 24°C and 30°C, then maintained low levels throughout the rest of the fermentation. The lactic acid bacteria level on day 15 was significantly higher in the crab sauces fermented at 24°C (6.3 ± 3.1 logCFU g^{-1}) and 30°C (8.5 ± 0.0 logCFU g^{-1}) than in the crab sauces fermented at 50°C (2.0 ± 0.0 logCFU g^{-1}) and 37°C (2.3 ± 0.2 logCFU g^{-1}). On day 90, the average bacterial population was 2.2 ± 0.2 logCFU g^{-1} with no significant differences between the fermentation temperatures.

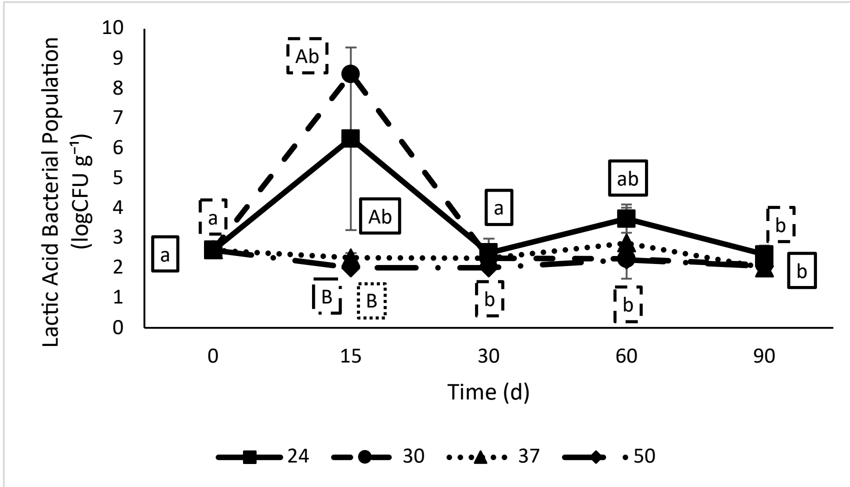

Figure 3.5. Lactic Acid Bacterial Population in Lab-Fermented Crab Sauces Over Time. Uppercase letters designate significant differences between treatments on individual testing days. Lowercase letters designate significant differences within treatment across time. The error bars represent the standard deviation. (n=3)

3.4.3. Non-Enzymatic Browning

Fermentation time and temperature exerted significant effects on the non-enzymatic browning. The non-enzymatic browning (Figure 3.6.) of lab-fermented crab sauce samples reached the highest level in all treatments on day 60 with the crab sauce fermented at 50°C (0.608 ± 0.07) significantly higher than crab sauce fermented at 24°C (0.173 ± 0.01). Higher fermentation temperatures corresponded to higher levels of non-enzymatic browning. This trend was still present in finished sauces at day 90, but there is a large margin of error for the crab sauce fermented at 37°C on day 90, which could obscure significant differences.

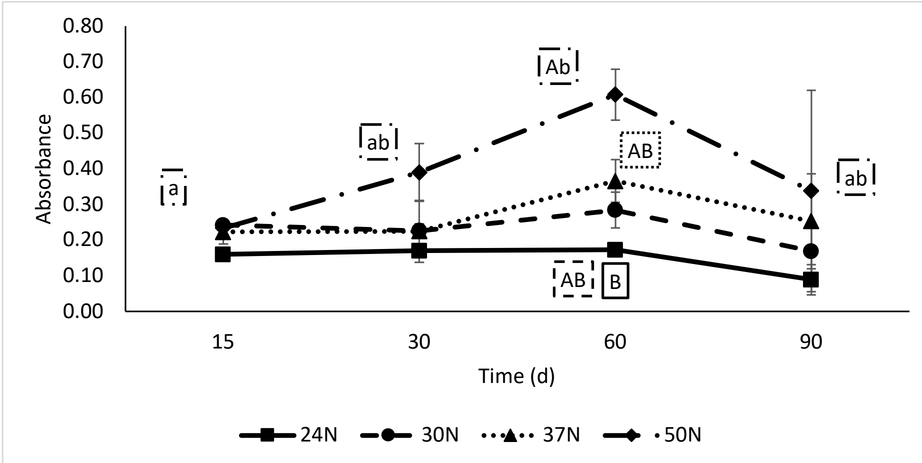

Figure 3.6. Non-Enzymatic Browning in Lab-Fermented Crab Sauces Over Time. Uppercase letters designate significant differences between treatments on individual testing days. Lowercase letters designate significant differences within treatment across time. The error bars represent the standard deviation. (n=3)

3.4.4. Amine Nitrogen

The fermentation time, fermentation temperature, and replicate all exerted significant effects on the amine nitrogen. The crab sauce fermented at 24°C had a significantly lower amine nitrogen content (Figure 3.7.) compared to 37°C throughout the entire fermentation. The amine nitrogen value for crab sauce fermented at 24°C (423.9 ± 57.2 mg N 100mL^{-1}) was significantly lower than those for crab sauces fermented at 30°C and 37°C (682.9 ± 62.2 mg N 100mL^{-1} and 679.8 ± 87.2 mg N 100mL^{-1} respectively) on day 90.

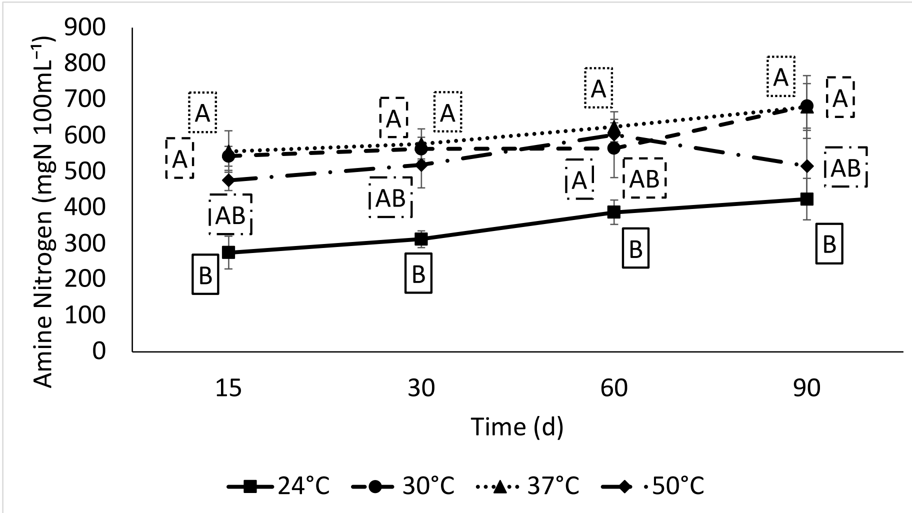

Figure 3.7. Amine Nitrogen Content in Lab-Fermented Crab Sauces Over Time. Uppercase letters designate significant differences between treatments on individual testing days. Lowercase letters designate significant differences within treatment across time. The error bars represent the standard deviation. (n=3)

3.4.5. Biogenic Amines

Fermentation time exerted a significant effect on total biogenic amines, but this effect did not appear to be linear. Cadaverine, putrescine, and tyramine were only identified sporadically in lab-fermented crab sauce samples (Table 3.1.). Cadaverine was only identified on two sampling occasions and putrescine was only identified on five sampling occasions. Of all amines identified histamine was present in the highest concentrations. The highest level of histamine identified at any point of fermentation and at any temperature was 10.37 ± 1.16 mg N 100 mL^{-1}, which is lower than the 50 mg N 100 mL^{-1} the FDA allows (Food and Drug Administration, 2019). Even total biogenic amines were substantially below this threshold at every sampling point. There were no significant differences in total biogenic amines due to fermentation time or temperature.

Table 3.1. Total Biogenic Amines in Lab Fermented Crab Sauces[1]							
Fermentation Temperature	Time (d)	Histamine (mg 100mL^{-1})	Agmatine (mg 100mL^{-1})	Putrescine (mg 100mL^{-1})	Cadaverine (mg 100mL^{-1})	Tyramine (mg 100mL^{-1})	Total Biogenic Amines (mg 100mL^{-1})
24°C	0	3.23 ± 0.36 a	2.04 ± 0.16	4.14 ± 0.05	N.D.	2.10 ± 0.10	11.51
	15	6.75 ± 0.30 ab	2.74 ± 0.29	N.D.	1.18 ± 0.08	N.D.	10.67
	30	7.37 ± 0.77 ab	4.04 ± 0.45	N.D.	N.D.	2.44 ± 0.03	13.85
	60	9.29 ± 0.83 b	4.06 ± 0.10	N.D.	0.89 ± 0.00	2.92 ± 0.00 A	17.16
	90	8.40 ± 1.03 b	4.00 ± 0.25	N.D.	N.D.	2.85 ± 0.09	15.25
30°C	0	3.23 ± 0.36 a	2.04 ± 0.16 a	4.14 ± 0.05	N.D	2.10 ± 0.10	11.51
	15	7.29 ± 0.98 ab	3.84 ± 0.25 ab	5.58 ± 0.92	N.D	N.D.	16.71
	30	10.37 ± 1.16 b	5.97 ± 0.18 b	N.D.	N.D.	2.32 ± 0.30	18.66
	60	7.85 ± 1.91 ab	4.29 ± 0.64 ab	N.D.	N.D.	2.40 ± 0.39 AB	14.54
	90	8.57 ± 0.99 b	4.93 ± 0.14 b	N.D.	N.D.	2.63 ± 0.17	16.13
37°C	0	3.23 ± 0.36 a	2.04 ± 0.16 a	4.14 ± 0.05	N.D.	2.10 ± 0.10	11.51
	15	7.02 ± 0.75 ab	5.23 ± 1.30 b	N.D.	N.D.	2.10 ± 0.20	14.35
	30	8.74 ± 0.34 b	5.15 ± 0.78 b	N.D	N.D.	2.18 ± 0.14	16.07
	60	7.91 ± 3.01 ab	4.20 ± 0.92 ab	N.D	N.D.	2.09 ± 0.50 AB	14.20
	90	8.22 ± 0.64 a	4.76 ± 0.78 b	N.D.	N.D.	2.25 ± 0.25	15.23
50°C	0	3.23 ± 0.36 a	2.04 ± 0.16 a	4.14 ± 0.05	N.D.	2.10 ± 0.10	11.51
	15	6.32 ± 1.15 ab	3.65 ± 1.06 ab	N.D.	1.31 ± 0.14	N.D.	11.28
	30	8.37 ± 0.22 b	4.31 ± 0.74 ab	N.D.	N.D.	2.02 ± 0.15	14.70
	60	7.03 ± 1.40 ab	4.64 ± 1.78 ab	N.D.	N.D.	1.81 ± 0.28 B	13.48
	90	9.31 ± 1.10 b	5.80 ± 1.42 b	N.D.	N.D.	2.40 ± 0.41	17.51
Uppercase letters designate significant differences between treatments on individual testing days. Lowercase letters designate significant differences within treatment across time. (n=3)							

3.5. Discussion

The mechanics of crab sauce fermentation rely on the degradation of protein due to proteolytic enzymes found within the crab viscera and those produced by microorganisms that grow and/or autolyse during the fermentation. This fermentation can be manipulated using various salt concentrations and fermentation temperatures. A concentration of 200-300 mg g^{-1} was previously found to produce a product comparable to commercially available fish sauce products based on measures of amine nitrogen, total volatile basic nitrogen, and water activity (Greiner et al., 2021). The fermentation temperatures used in the present study were chosen as approximately ambient temperature (24°C), temperatures used in previously published studies investigating fish fermentation (30°C and 37°C; Aquerreta et al., 2002; Zhou et al., 2016), and optimal for proteolytic enzymatic activity in fish sauce fermentation (50°C; Lopetcharat & Park, 2002).

In previous studies employing temperatures from 24-37°C, pH was found to increase throughout the course of the study (Jung et al., 2016; Kilinc et al., 2006; Zhou et al., 2016) as was amine nitrogen, which has been tied to desirable flavors and is generally equated to fermentation progress as a measure of proteolysis (Wang et al., 2018). All of the fish sauces fermented at the different temperatures showed an increase in the amine nitrogen over time, except for fish sauce fermented at 50°C. At the higher fermentation temperatures of 30°C and 37°C, a peak in the bacterial growth early in the fermentation was followed by a decrease throughout the rest of the fermentation time (Kilinc et al., 2006; Zhou et al., 2016), as seen at temperatures of 24°C, 30°C, and 50°C throughout this study on all media. Previous fish sauce fermentation processes at 37°C have also indicated an increased reliance on endogenous enzymatic activity as opposed to strictly bacterial growth (Aquerreta et al., 2002), which partially explains why despite the crab sauce fermented at 37°C not exhibiting a peak in bacterial growth early in the fermentation, fermentation demonstrably proceeded as measured by amine nitrogen content.

After research into previous studies, higher temperatures would be expected to produce higher levels of enzymatic activity and protein degradation. At all temperatures, previous research has shown a sharp increase in aerobic bacterial population followed by a consistent decrease, which would be expected in this study as well and was observed in all media at all temperatures except 37°C. Our data indicated that the highest level of proteolysis, as identified through amine nitrogen, occurred at 30-37°C, with notable levels of proteolysis occurring at 50°C as well.

In fish sauce fermentation studies employing lactic acid bacterial starter cultures, a decrease in pH is typically observed due to the production of lactic acid (Saithong et al., 2010). In studies investigating a more traditional, spontaneous fermentation, including limited ingredients such as salt and fish, the pH has been consistently found to increase mainly due to the production of biogenic amines and other volatile bases tied to the putrefaction of the starting material (Aquerreta et al., 2002; Kilinc et al., 2006; Zhou et al., 2016). In this study, there was an increase in the pH of the crab sauce fermented at 50°C (maximum of 7.83 ± 0.34 on day 90). The pH of the crab sauce fermented at lower temperatures of 24°C, 30°C, and 37°C did not significantly decrease over time, but did show a negative trend over the course of the fermentation.

Water activity is a measure of the water in a food available for biological activity that can be an indication of the stability or food safety of the product (Kilinc et al., 2006). A low water activity can be used to prevent microbial growth at a widely accepted level of 0.9 (Department of Health, Education, and Welfare Public Health Service 2014), but in order to protect foods against spoilage by extremophilic microbial species a water activity closer to 0.632 is recommended (Stevenson et al., 2015). If a water activity of 0.85 or lower is maintained in the final product, the product is not subject to food safety regulations regarding water activity (Department of Health, Education, and Welfare Public Health Service 2014). The crab sauce fermented at all temperatures maintained a water activity well below the regulated level of 0.85, indicating a safe product unlikely to harbor common pathogens, but potentially subject to spoilage by extremophiles. The crab sauce fermented at 24°C had a higher level of water activity (an average of 0.762 ± 0.005 over the course of the fermentation) compared to sauces fermented at higher temperatures, but was still below the regulated level.

All of the bacteria investigated – proteolytic bacteria, histamine-forming bacteria, and lactic acid bacteria – followed trends similar to those of previously published studies, peaking early in the fermentation and then maintaining a lower population throughout the rest of the timepoints. The only temperature that did not result in a peak on day 15 of fermentation was the crab sauce fermented at 37°C. The similarity of bacterial trends for crab sauces fermented at 24°C-37°C regardless of the type of bacteria indicates the same population is being investigated by all the media. For the crab sauce fermented at 50°C, there is no peak in lactic acid bacteria on day 15, implying that all of the bacterial counts are not investigating the same population. These trends of low bacterial population later in the fermentation are also consistent with trends in the literature (Aquerreta et al., 2002; Kilinc et al., 2006; Lopetcharat & Park, 2002; Zhou et al., 2016) most likely due to consumption of existing nutrients. These trends were not mirrored in any of the chemical properties of the fermented crab sauce.

The non-enzymatic browning is primarily a measure of browning due to the Maillard reaction which involves reducing sugars, oxidation products, and amino acids (Lopetcharat et al., 2001; Zhao et al., 2017). The non-enzymatic browning in this study showed an increasing trend at fermentation temperatures of 37°C and 50°C, similar to trends found in fish sauce fermented at ambient temperature (Peralta 2010), and was highest in the crab sauce fermented at 50°C. The non-enzymatic browning was lowest in the crab sauce fermented at 24°C, which aligns with the amine nitrogen results presented in this study.

The release of nitrogenous compounds is one of the main mechanisms of the spontaneous fermentation, quantified partially through measurement of amine nitrogen (Ijong & Ohta, 1996). The amine nitrogen had a positive trend over time at all fermentation temperatures and was lowest in the crab sauce fermented at 24°C at all timepoints of the fermentation. Amine nitrogen levels have been associated with desirable flavors in fish sauce (Lopetcharat et al., 2001; Zhao et al., 2017), making a high level of amine nitrogen favorable in the fermentation of a seafood condiment. The amine nitrogen contents of crab sauces fermented at 30 and 37°C were the highest at all time points of the fermentation, suggesting these fermentation temperatures produced a more flavorful product, based on the chemical makeup. It is conceivable that these values would continue to increase with extended fermentation, so a longer duration may be worthy of investigation. Globally, some fish sauces are fermented for as long as 12 months (El Sheikha & Montet, 2014). Previous research has been conducted to identify the chemical targets for a crab sauce as based on commercially available fish sauce products (Greiner et al., 2021). These targets include values for pH, water activity, amine nitrogen, non-enzymatic browning, and biogenic amines. None of the tested temperatures reached a value comparable to the pH, amine nitrogen, agmatine, cadaverine, putrescine, or histamine in the commercial samples. The water activity on the final day of fermentation for crab sauces fermented at 30°C and 37°C and the tyramine content for all fermentation temperatures were comparable to the commercial samples.

In some instances, the nitrogenous compounds released by the proteolysis are decarboxylated into biogenic amines, the most worrisome being histamine. High levels of histamine, when ingested by humans, can cause rhinoconjuctival symptoms, hypotension, diarrhea, and headache (Maintz & Novak, 2007). Histamine levels in the United States are regulated to be no more than 50 mg 100 mL^{-1} in foods (Food and Drug Administration, 2019). In this study, the histamine levels were found to be below the legally allowed limit in the United States at all fermentation temperatures, suggesting that there is unlikely to be a chemical hazard associated with the product regardless of process variation.

Based on the amine nitrogen content, the water activity, and the legally abiding histamine content, the recommended temperature range for the fermentation of *Carcinus maenas* is 30°C to 37°C.

There is very little data on consumer acceptability of fish sauce and no data on the consumer acceptability of a fermented crab sauce. Additional investigation should include the consumer testing of a fermented crab sauce condiment to understand acceptable characteristics of a product and consumer acceptability of current fermentation methods, as well as shelf life testing to identify potential spoilage issues.

3.6. Conclusion

The fermentation of *Carcinus maenas* provides an opportunity for both an economic and ecological benefit from control of an invasive species. Such a fermentation can be achieved through combining crab and salt which is then fermented for 90 days or longer. Although a temperature of 30°C to 37°C produces a condiment with a favorable chemical makeup and stability, a temperature of 24°C or 50°C could also be used to produce a fermented crab condiment. In order to ensure good market opportunity, consumer surveys and testing must be investigated. This optimization of fermentation through temperature control provides a more efficient alternative to fermenting *Carcinus maenas* than prior research, offering a more economically friendly method by offering multiple fermentation temperatures and the outcomes of such a fermentation.

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3.9. Author Contributions

Delaney M Greiner: Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Visualization **Denise I. Skonberg:** Conceptualization, Methodology, Data Curation, Writing – Review & Editing, Supervision, Funding Acquisition **L. Brian Perkins:** Methodology, Data Curation, Writing – Review & Editing **Jennifer J. Perry:** Conceptualization, Methodology, Data Curation, Writing – Review & Editing, Supervision, Funding Acquisition

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CHAPTER 4

EVOLUTION OF MICROBIAL CONSORTIA DURING FERMENTATION OF *CARCINUS MAENAS* AT VARIOUS TEMPERATURES

4.1. Abstract

The fermentation of *Carcinus maenas* into a crab condiment serves as a high value method to incentivize fishing of invasive crabs to minimize damage to the soft-shell clam industry. Crabs were trapped off the coast of Maine, crushed in a sausage grinder, combined with 200mg g^{-1} of salt and fermented for up to 90 days at temperatures of 24°C, 30°C, 37°C, and 50°C. DNA was extracted from the resulting sauce and was subjected to 16S amplicon sequencing. The data were analyzed using R studio for trimming, alpha diversity, and beta diversity. No significant differences were identified in the alpha diversity due to temperature or fermentation time. The most abundant families identified were *Spirobacteracea, Hyphomonadaceae,* and *Rhodobacteracea*, consistent with high levels of *Firmicutes* identified early in fish sauce fermentation. The pre-fermented whole crab had an abundance of *Lactococcus* bacteria. The data show that the bacterial populations present for fermentation are not dependent on temperature or fermentation time, identifying three dominant microbial clades in high abundance in all treatments and timepoints. This indicates that fermentation temperature does not change the bacterial mechanics of the fermentation.

4.2. Introduction

The European green crab, *Carcinus maenas*, is an invasive species that has caused both economic and ecological turmoil on the North American East Coast. The green crabs uproot eelgrass beds, disrupting ecosystems along the coast (Polte et al., 2005), and prey on bivalve mollusks (Rangeley & Thomas, 1987), causing immense losses in the soft-shell clam industry (Congleton et al., 2006).

Unfortunately, these crabs only average a carapace width of about 79.9 ± 3.9 mm (Skonberg & Perkins, 2002), so they cannot be picked for meat like commercially important crabs such as Jonah and blue crabs (NOAA Fisheries, 2021; Truesdale, 2018), necessitating the identification of alternative profitable uses. Soft-shell green crab fisheries have been established on the North American East Coast, but produce a large amount of biomass that can currently be sold as compost or animal feed (Department of Marine Resources, 2014), both low-value valorization opportunities. In order to incentivize fishers to trap green crabs, high-value opportunities need to be established.

Previous research has explored a high-value option for waste valorization by fermenting the green crab biomass into a crab sauce condiment comparable to fish sauce, a brown, fish-smelling condiment traditionally used in South Asian cooking now available world-wide (El Sheikha & Montet, 2014; Lopetcharat et al., 2001). Research has indicated an optimal salt content of 200mg g^{-1} and a suggested fermentation temperature within the range of 30°C to 37°C (Greiner et al., 2021). A recent survey identified a high-level of interest in this product from chefs, resulting in statements such as "Great way to solve a problem," and "Having a local Maine product—that also helps with an invasive species—would be a dream" (Leung et al., unpublished data)

Due to the unique and novel nature of this product, many of the aspects of the fermentation are not well understood. Despite the availability of literature characterizing bacterial populations in traditional fish sauces (Du et al., 2019; Faisal et al., 2015), which have shown peaks of bacterial growth early in the fermentation (Zaman et al., 2011), differences in raw material are expected to result in distinct microbiota. In prior studies, populations of proteolytic, lactic acid bacteria, and a total plate count (Greiner et al., 2021) have been quantified with a sharp increase in bacterial population early in the fermentation with a subsequent decline. The purpose of this work was to investigate the bacterial taxa actively partaking in fermentation at different time points and fermentation temperatures throughout the fermentation of a green crab-based condiment.

4.3. Materials and Methods

4.3.1. Crab Fermentation and Crab Sauce Collection

Green crabs were caught on the coast of Maine and transported on ice to the University of Maine (Orono, ME), frozen at -20°C until dead and stored at -20°C until use. The whole, frozen crabs were thawed for 36-48 hours at 4°C. Crabs were finely ground in a meat grinder (Hobart Corporation, Troy, OH) using a 3/16 inch die and combined with 200 mg g^{-1} (w/w) canning and pickling salt (Morton Salt, Chicago, IL). All crab was prepared for fermentation at four separate temperatures in triplicate in 0.95 L glass canning jars (Ball Corporation, Westminster, CO) containing 800g of crab and salt mixture covered with a double layer of cheesecloth (Pyrm Consumer USA, Spartanburg, SC).

The crab and salt mixture was incubated at one of four temperatures (24°C, 30°C, 37°C, 50°C) for up to 90 days. After 15, 30, 60, and 90 days of fermentation one jar of each treatment per replicate was used for analysis (no repeated sampling). The sauce was separated from the solid particulate by filtering through two layers of cheesecloth into 250 mL centrifuge tubes. On day 90, the solid particulate and sauce were poured into a 500 mL centrifuge tube. Tubes were centrifuged in an Avanti J-E Beckman Coulter centrifuge (Brea, CA) (150 x *g*, 10 min). The supernatant was collected for DNA extraction and purification.

4.3.2. DNA Extraction and Purification

The microbial DNA present in the fish sauce was extracted using the DNeasy PowerFood Microbial Kit (Germantown, MD) according to manufacturer instructions. The extracted DNA was purified for analysis using the GeneJET Genomic DNA Purification Kit (Waltham, MA), also according to manufacturer instructions. DNA from replicate treatment/time combinations were pooled prior to amplification.

4.3.3. PCR Amplification

Amplification and sequencing were performed on the V4 region of the 16S rRNA gene. Samples were barcoded and primers 515f and 806R (Wang et al., 2018; Wang et al., 2021) were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD). The following thermal profile was used: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute. Then the final elongation step was performed at 72°C for 10 minutes, resulting in a 300 base pair amplicon. Post amplification, the products from the PCR process were visualized on a 2% agarose gel to determine the success of the amplification by the intensity of the bands.

The samples were multiplexed using unique indices and were pooled together in equal proportions depending on molecular weight and DNA concentration. These pooled samples were purified using calibrated Ampure XP Beads (Beckman Coulter, Indianapolis, IN). This PCR product was used to prepare an Illumina DNA library. Sequencing was performed at MR DNA (Shallowater, TX, USA) on a MiSeq (SanDiego, CA) following the manufacturer's guidelines.

4.3.4. Analysis of Sequencing Data

The analysis of the amplicon sequencing data followed a pipeline used in Ishaq et al. (2020). Sequenced DNA was analyzed using R Version 4.0.3 (Boston, MA). An installation package called BiocManager (Biocversion, 2021) was installed to aide in the installation of all other packages. The packages dada2 (Callahan et al., 2016), GenomeInfoDBData (Bioconductor Core Team, 2020) and phyloseq (McMurdie & Holmes, 2013) were installed for the trimming, reading, and removal of chimeras from the data. The samples were read into the program by the package ShortRead (Morgan et al., 2009) to contain 6619915 reads and 35 and 251 cycles for both forward and reverse samples. Ten bases were trimmed from the front and back of each read both forward and reverse. The samples were found to have 2887 bimeras out of 10684 total sequences. These bimeras were removed. After checking the quality, trimming, and removing chimeras, 17 samples representative of times and treatments tested remained with 7797 sequence variants (SVs). The sequences, meta data, and Silva version 138 taxonomic data (McLaren, 2020) were compiled into a sequence table showing the presence of 7797 taxa, 17 samples, 9 sample variables, and 7 taxonomic ranks. Contaminants were removed using the package decontam (N. M. Davis et al., 2017), leaving the data with 7721 taxa, 17 samples, 9 sample variables, and 7 taxonomic ranks. Chloroplasts and mitochondria were cleaned out with the package dplyr (Wickham et al., 2021). This was the final step of quality analysis, leaving 5622 taxa, 17 samples, 9 sample variables, and 7 taxonomic ranks for further analysis.

The remaining data were then rarified for better comparison using the package vegan (Oksanen et al., 2020). The sample size was maxed at 9000 SVs and 1106SVs were removed. The set seed used for random sampling was 711. The package ggplot2 (Wickham, 2016) was used to build alpha diversity plots. The package phyloseq was used to measure alpha diversity. A Shapiro-Wilkes test was used to determine the normality of the alpha diversity data. Normal data were analyzed for variance using an ANOVA test and pair-wise comparisons were analyzed with a Tukey's Honestly Significant Difference (HSD) test. Non-normal data were analyzed for variance using a Wilcox Test and pair-wise comparisons were analyzed with a pair-wise Wilcox Test.

The 100% abundance bar plot was made using the ggplot2, phyloseq, and tidyverse (Hadley Wickham et al., 2019) packages. The sample counts were transformed to calculate abundance. The data frame was melted, taxa of less than 1% were grouped into a "<1% Abundance" group, and any genera appearing as "NA" were replaced with family designation. The plot was formed using the ggplot function.

Beta diversity was investigated using a Principle Coordinate Analysis (PCoA) plot. This was built using phyloseq, vegan and ggplot2 packages. The Bray-Curtis ordination was calculated using the ordinate function. The data was plotted using the package viridus (Citation). Significance was identified using a permanova test with the adonis function.

4.4. Results

The Shannon alpha diversity (Figure 4.1) is a measure of bacterial richness. There were no significant differences due to fermentation temperature. The Shannon bacterial richness due to the sampling day ranged from about 5.0 to 5.50 SVs. The diversity significantly increased from day 15 to day 30 and peaked on day 30. The Shannon bacterial richness significantly decreased from day 30 day 90.

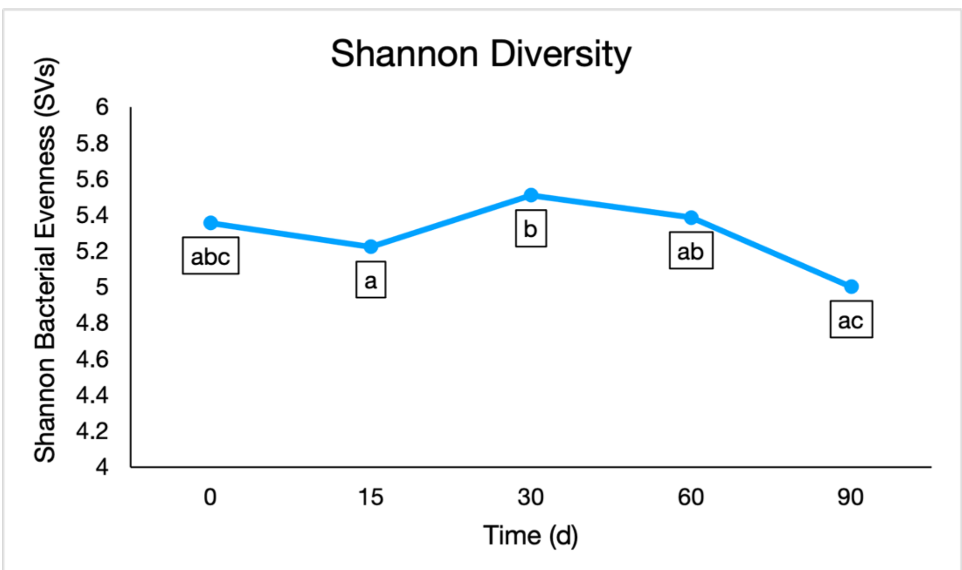

Figure 4.1. Shannon Alpha Diversity of Microbiota in Lab-Fermented Crab Sauces as Affected by Fermentation Temperature and Time. Lowercase letters designate significant differences over time. (n=4)

The observed alpha diversity (Figure 4.2.) is a measure of bacterial richness. The fermentation temperature that produced the highest bacterial richness is 30°C, at approximately 875 SVs and the fermentation temperature with the lowest bacterial richness was the lowest temperature, 24°C with only 750 SVs of observed bacterial richness. This is a high level of bacterial richness for a fermented seafood condiment. Previous studies have identified a range of 254 ± 70 to 763 ± 36 SVs as a range of bacterial richness in fish sauce (Du et al., 2019). There were no significant differences in the observed microbial diversity due to fermentation time or temperature.

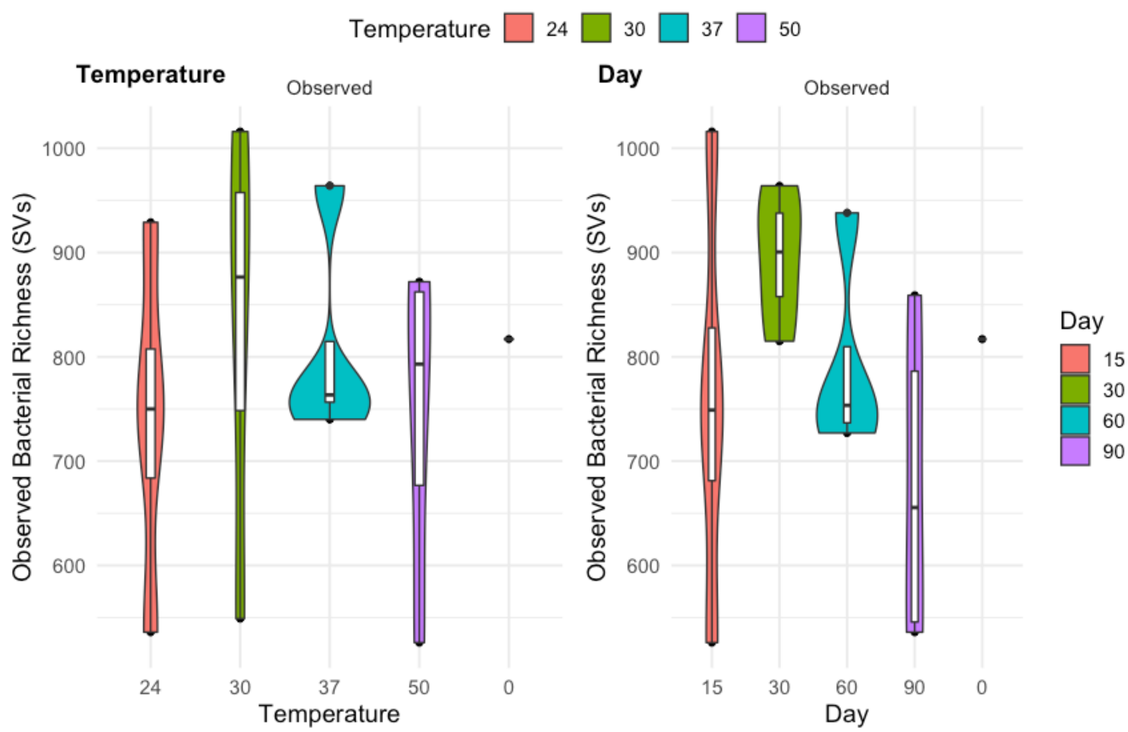

Figure 4.2. Observed Alpha Diversity of the Microbiota in Lab-Fermented Crab Sauces as Affected by Fermentation Temperature and Time. (n=4)

The beta diversity (Table 4.1.) is a measure of diversity between the different treatments as a whole rather than the individual sampling instances. There appears to be a high level of diversity between the whole crab starting material that was tested before being fermented and the fermented crab sauces. The bacterial diversity differences between the pre-fermented crab and the crab sauce increase as the temperature increases when the designated fermentation temperature is applied. Based on the results from the beta diversity, with the diversity between fermented samples and the pre-fermented crab increasing with the application of fermentation temperature, and the results of the alpha diversity, showing the bacterial richness in the fermented crab has an average lower than the pre-fermented crab.

Table 4.1. Beta Diversity of Fermented Crab Sauce as Affected by Temperature and Time

	CrabSauce-24-15	CrabSauce-24-30	CrabSauce-24-60	CrabSauce-24-90	CrabSauce-30-15	CrabSauce-30-30	CrabSauce-30-60	CrabSauce-30-90	CrabSauce-37-15	CrabSauce-37-30	CrabSauce-37-60	CrabSauce-37-90	CrabSauce-50-15	CrabSauce-50-30	CrabSauce-50-60	CrabSauce-50-90
CrabSauce-24-30	0.322															
CrabSauce-24-60	0.416	0.449														
CrabSauce-24-90	0.424	0.399	0.502													
CrabSauce-30-15	0.258	0.319	0.414	0.451												
CrabSauce-30-30	0.403	0.327	0.485	0.451	0.370											
CrabSauce-30-60	0.424	0.362	0.436	0.440	0.392	0.361										
CrabSauce-30-90	0.399	0.340	0.461	0.337	0.385	0.336	0.299									
CrabSauce-37-15	0.301	0.312	0.416	0.438	0.284	0.360	0.369	0.348								
CrabSauce-37-30	0.417	0.366	0.448	0.446	0.393	0.369	0.309	0.327	0.348							
CrabSauce-37-60	0.423	0.377	0.434	0.452	0.402	0.374	0.310	0.307	0.357	0.324						
CrabSauce-37-90	0.401	0.368	0.457	0.342	0.395	0.362	0.329	0.234	0.361	0.346	0.317					
CrabSauce-50-15	0.454	0.441	0.438	0.462	0.451	0.464	0.449	0.434	0.430	0.460	0.448	0.440				
CrabSauce-50-30	0.428	0.361	0.469	0.362	0.410	0.414	0.328	0.342	0.396	0.379	0.375	0.365	0.409			
CrabSauce-50-60	0.421	0.358	0.425	0.444	0.401	0.364	0.287	0.296	0.363	0.314	0.215	0.318	0.439	0.352		
CrabSauce-50-90	0.361	0.312	0.414	0.360	0.352	0.371	0.342	0.308	0.313	0.373	0.351	0.300	0.398	0.292	0.336	
WholeCrab-0	0.640	0.598	0.597	0.615	0.626	0.635	0.614	0.649	0.623	0.642	0.646	0.652	0.611	0.558	0.633	0.591

The PCoA (Figure 4.3.) does not indicate a high level of clustering, with a large downward spread. This is indicative of an effect from both fermentation time and temperature because there is more than one fermentation temperature or time deviating from a cluster. This was confirmed by a permanova.

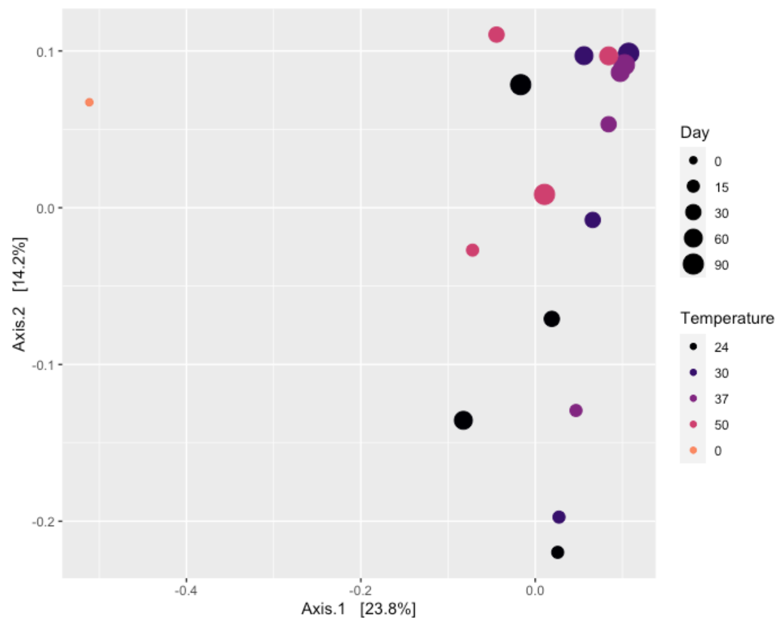

Figure 4.3. PCoA as a Measure of Beta Diversity in Lab-Fermented Crab Sauce Samples as Affected by Fermentation Temperature and Time. (n=1, representing 3 pooled replicate samples)

Based on the permanova output (Table 4.1.), there are significant differences in the beta diversity due to fermentation temperature and sampling day, but not the interaction of fermentation temperature and sampling day.

Table 4.2. Permanova Output for Bray-Curtis Measure of Diversity

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Temperature	4	0.55269	0.138172	2.02647	0.39506	0.0069
Day	1	0.12770	0.127700	1.87289	0.09128	0.0176
Temperature:Day	3	0.17312	0.057708	0.84636	0.12375	0.7046
Residual	8	0.54547	0.068183		0.38990	
Total	16	1.39898			1.39898	

The three most abundant families of bacteria present in crab sauce samples were identified to be *Saprospiraceae*, *Hyphomonadaceae, and Rhodobacteraceae* (Figure 4.4.). There were no consistent trends related to time or temperature. The whole crab starting material that was collected on day 0 does have a noticeably higher abundance of *Streptococcaceae,* but levels of this genus decreased in subsequent samplings.

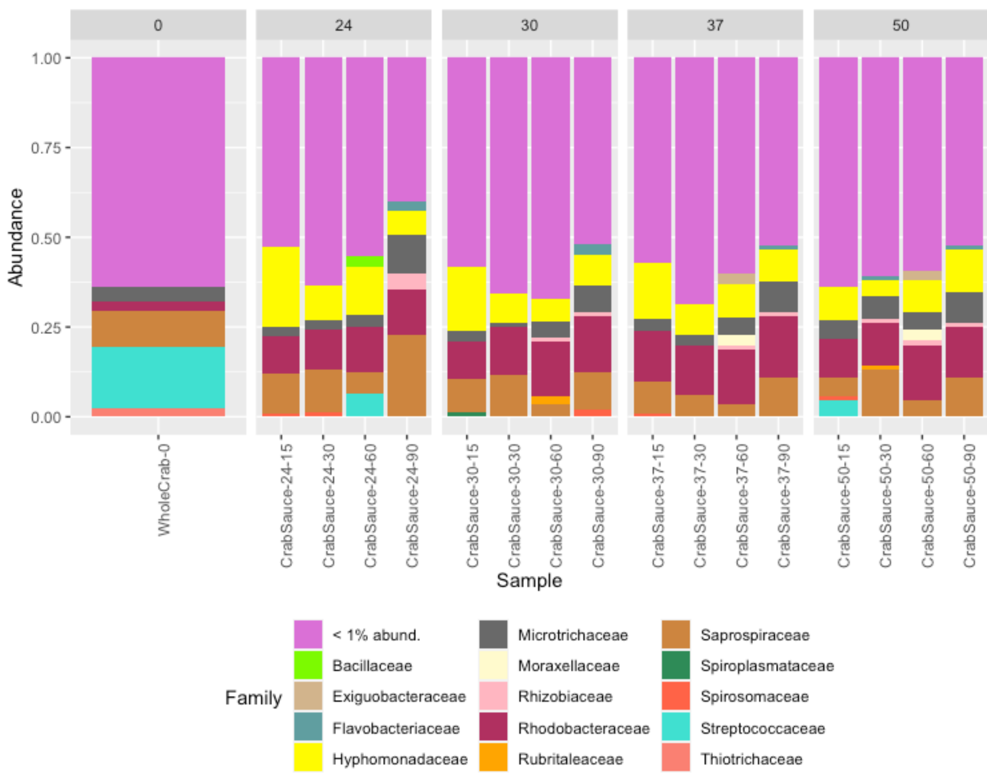

Figure 4.4. Abundance of Families/Genera in Lab-Fermented Crab Sauces.

[a]The <1% abundance group is comprised of 266 families.

4.5. Discussion

The fermentation used to produce a crab sauce product is a spontaneous fermentation relying on the activity of proteolytic enzymes. Similar to the process used to ferment fish sauce, these enzymes come from the viscera of the product being fermented, in this case green crab, and from the microorganisms found on the product being fermented (Orejana & Liston, 1982). The bacteria typically responsible for the production of proteolytic enzymes in this fermentation are also typically halophiles because of the high salt content associated with fish sauce (Xiao et al., 2014).

The diversity of the bacteria present can be looked at within each sample or between the samples. The alpha diversity is an indicator of bacterial richness and evenness within each sample that is being investigated. Two different methods of investigating alpha diversity include the Shannon diversity, investigating the distribution and representation of the species, and the Observed diversity (CHAO), investigating the entropy and the rarity of the species present (Du et al., 2019). The Shannon diversity observed in crab sauce samples significantly increased and then decreased over the course of the fermentation with increasing fermentation time regardless of temperature suggesting that bacteria from the *Saprospiracea* family grew faster than others and then slightly declined, decreasing the richness of bacteria. The Observed alpha diversity was not significantly influenced by either fermentation temperature or time, which suggests that the same bacteria were present throughout the course of the fermentation regardless of time or temperature.

The beta-diversity was measured to identify the microbial differences between all of the crab sauces collected, including the whole crab on day 0, prior to fermentation. Since there is a high differential in the diversity of the whole crab and the fermented crab sauce, this leads to the understanding that different microorganisms, primarily those from the family *Saprospiraceae, Rhodobacteraceae,* and *Hyphomondaceae*, are fostered to grow throughout the fermentation that aren't in high abundance in the green crab. These families of microorganisms can out compete other families of bacteria, such as *Streptococcaceae*, lowering the bacterial diversity.

The bacterial richness in this study was very high compared to other literature with a Shannon alpha diversity of 3.662 (Du et al., 2019) in traditional Chinese fish sauce, showing that crab sauce fermentation employs a higher diversity of microorganisms and an almost completely even amount of these microorganisms. Beta diversity, an indication of diversity between the actual samples, as defined by the Bray-Curtis approach, is based on the abundance of amplicons assigned to each taxa in each sample.

The abundance plot focused on the abundance on a scale of 100%. The abundance plot shows few differences over time or between fermentation temperatures, other than high levels of *Streptococcaceae* in the starting whole crab. *Lactococcus* is a genus in the family *Streptococcaceae* typically expected to be seen in fermented products (Cavanagh et al., 2015; Silpa & Rupachandra, 2020; Wang et al., 2020), included in fish sauce fermentation. This genus has been found in gastrointestinal tracts of other aquatic animals (Mortezaei et al., 2020). Its abundance, however, decreased markedly after salting, since *Lactococcus* typically only tolerates a salt concentration of 2.5-4% (Sanders et al., 1999).

The three most abundant families found in the fermented crab sauce samples were *Saprospiraceae, Hyphomonadaeceae,* and *Rhodobacteraceae.* These most abundant families have all been isolated from marine environments and have been documented to possess proteolytic activity in at least one species within the family (Durighello et al., 2014; Lei et al., 2014; White et al., 2019). *Saprospiraceae* additionally has been found in a large variety of habitats, being found in a marine environment isolated from sea sponges and algae, and also requires the presence of salt for growth while being able to grow with a salt content of 7% in culture media (Rosenberg et al., 2014; Xia et al., 2008). *Hyphomonadaeceae* includes a majority of genera that have been identified from a marine environment with some species that can tolerate salt up to 10% (Rosenberg et al., 2014). The family *Rhodobacteracea* contains bacteria identified in marine environments, commonly requiring a salt content of 2-6% for optimal growth (Rosenberg et al., 2014), Since the fermentation used to produce a fish sauce product is spontaneous, the microbiota varies depending on the starting material used. Different marine species have drastically different diets, which contributes to dramatically variable endogenous enzyme activity and microbiota. Anchovies, commonly used in fish sauce fermentation, tend to eat *Euterpina acutifrons,* copeopds, and fish larvae (Bacha & Amara, 2009; Raab et al., 2011). Green crabs tend to prey on bivalve mollusks and eelgrass (Davis et al., 1998; Malyshev & Quijón, 2011; Rangeley & Thomas, 1987). The primary organisms responsible for the fermentation of fish sauce are *Bacillus* sp. and *V. halodenitrificans* (Xiao et al., 2014), both from the *Bacillaceae* family. These organisms primarily found in fish sauce were also consistently identified in the lab-fermented crab sauce in the abundance plot but not at the highest abundance.

Despite differences in temperature and fermentation time, the same bacterial families and genera were active throughout the course of the fermentation. More research needs to be done with sensory characteristics to determine guidance regarding fermentation temperature based on consumer preferences regarding taste in culinary applications. Modulation of endogenous bacteria by application of starter cultures may be a useful strategy should sensory characteristics be considered unacceptable by consumers in future research. *Staphylococcus carnosus* and *Tetragenococcus halophilus* were investigated as potential starter cultures and showed little benefit, most likely due to the death of the *Streptococcacea* family of bacteria during fermentation. *Halobacterium* sp. SP1(1), a commercial starter culture that has a high salt tolerance and has been found to positively impact flavor and aroma in fish sauce (Akolkar et al. 2010), could be used to improve sensory characteristics if necessary.

4.6. Conclusion

Carcinus maenas was primarily fermented by the endogenous families *Saprospiraceae, Hyphomonadaceae,* and *Rhodobacteraceae.* Although the fermentation was found to use different bacteria than those reported for fish sauce, both fermentations were driven by salt tolerant, proteolytic bacteria. Consumer testing is called for to identify differences in sensory characteristics resulting from utilization of different fermentation temperatures. These findings help to guide producers by identifying key bacterial groups that could be included as starter cultures, or offering multiple fermentation temperatures that follow a similar fermentation process based on the bacterial populations that were identified in the resulting sauces and the lack of diversity within the sauces regardless of fermentation time and temperature.

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4.9. Author Contributions

Delaney M. Greiner: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Denise I. Skonberg:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Jennifer J. Perry:** Conceptualization, Data curation, funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing

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APPENDIX A

RESULTS FROM SEPARATION OF RAW MATERIAL STREAMS AND INCLUSION OF STARTER CULTURES

A.1. Summary

Other studies were done in partial fulfillment of this thesis that did not give statistically significant results of interest. These studies investigated the separation of green crab raw material streams prior to fermentation, the inclusion of *Staphylococcus carnosus* as a starter culture, due to its reported ability to prevent the formation of histamine in fish sauce (Zaman et al., 2014), and the inclusion of *Tetragenococcus halophilus* as a starter culture, due to its ability to reduce biogenic amine content in fish sauce (Udomsil et al., 2011).

The separation of the mince and the shell was accomplished using a Paoli deboner, with a standardized salt content of 20% (w/w). The whole crab was prepared similarly to the studies outlined in chapters two and three. The collection of the crab sauce followed the same procedure as used in chapters two and three. The resulting sauce was tested for proteolytic bacteria, lactic acid bacteria, *Bacillus* spp., total plate count, pH, water activity, amine nitrogen, and biogenic amines. Statistics were run on this data, which again followed the same procedure documented in chapters two and three.

The inclusion of the starter cultures both followed a similar process. The starter cultures were grown in broth and the whole ground crab was inoculated at a level of 10^6 CFU g^{-1}. Crab was prepared for fermentation at temperatures of 30°C (fermentation with *T. halophilus* and control) and 37°C (fermentation with *S. carnosus* and control). The starter cultures were added before salting the crab and starter culture mixture. The collection of the resulting crab sauce followed the same procedure as outlined in chapters two and three. The sauce was tested for proteolytic bacteria, lactic acid bacteria, pH, water activity, non-enzymatic browning, amine nitrogen, and biogenic amines. The crab sauces fermented with separate streams was also tested for total plate count bacteria, using the same method in chapter two, and *Bacillus* spp. which was plated on mannitol yolk polymyxin (HiMedia, West Chester, PA; incubated at 30°C for 48h). The crab sauces fermented with starter cultures were also tested for histamine forming bacteria with the same media used in chapter three and mannitol fermented staphylococci which was plated on mannitol salt agar (Criterion Scientific, Atlanta, GA; incubated at 37°C for 48h). Statistics were run on this data, which also followed the same procedure documented in chapters two and three.

These methods of fermentation produced results that indicated that fermenting whole crab (as opposed to mince or shell) and fermenting without starter cultures was the best method moving forward for the sake of simplicity based on the lack of beneficial effect of stream separation and starter culture addition. The results are depicted and explained below.

A.2. Separation of Streams Results

The proteolytic bacterial population (Figure A.1.) in whole crab measured on day one was significantly higher than all other timepoints, reaching of 8.7 ± 0.0 logCFU g^{-1} compared to an average of 2.4 ± 0.4 logCFU g^{-1} at the rest of the timepoints. The whole crab had a significantly higher proteolytic bacterial population on day one compared to the shell crab and mince (8.7 ± 0.0 logCFU g^{-1}, 3.6 ± 0.0 logCFU g^{-1}, and 3.0 ± 0.0 logCFU g^{-1} respectively). A higher proteolytic bacterial population was tied to higher levels of proteolysis, which was the primary mechanism of the fermentation.

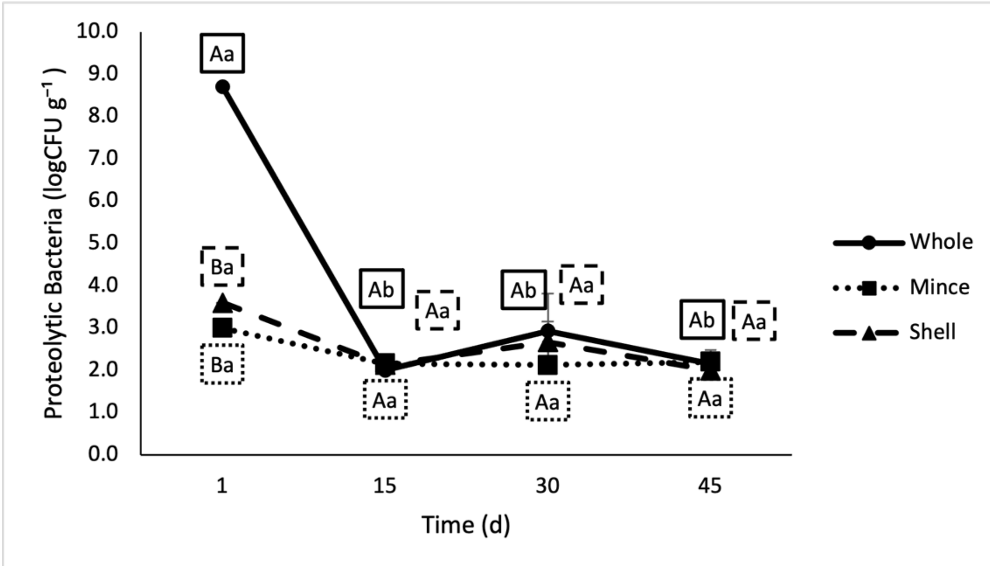

Figure A.1. Proteolytic Bacterial Population of Crab Sauces Fermented from Separated Streams Over Time. The lowercase letters designate significant differences over time. The uppercase letters designate significant differences among treatment. The error bars represent the standard deviation. (n=3)

The total plate count of bacterial population (Figure A.2.) significantly decreased after day one from an average of 4.4 ± 0.1 logCFU g^{-1} to 2.1 ± 0.1 logCFU g^{-1} across all treatments. No difference due to stream separation was observed.

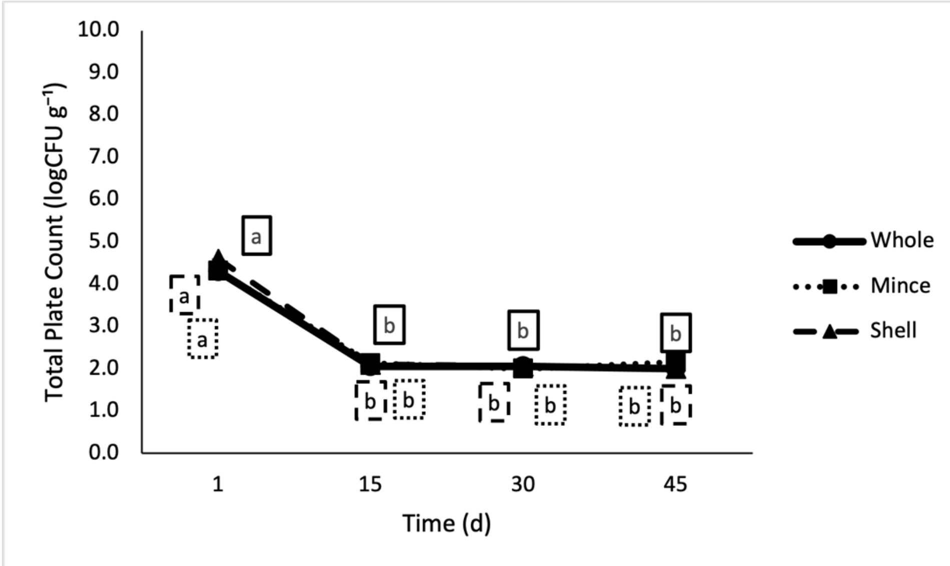

Figure A.2. Total Plate Count of Crab Sauces Fermented from Separated Streams Over Time. The lowercase letters designate significant differences over time. The error bars represent the standard deviation. (n=3)

The crab mince started with a significantly larger lactic acid bacterial population (Figure A.3.) than whole and shell (8.8 ± 0.0 logCFU g^{-1}, 6.7 ± 0.0 logCFU g^{-1}, 6.3 ± 0.0 logCFU g^{-1} respectively) on day one. The lactic acid bacterial population significantly decreased among all treatments after day one and maintained no significant changes over the remaining time points, resulting in an average population among all treatments of 2.0 ± 0.0 logCFU g^{-1}.

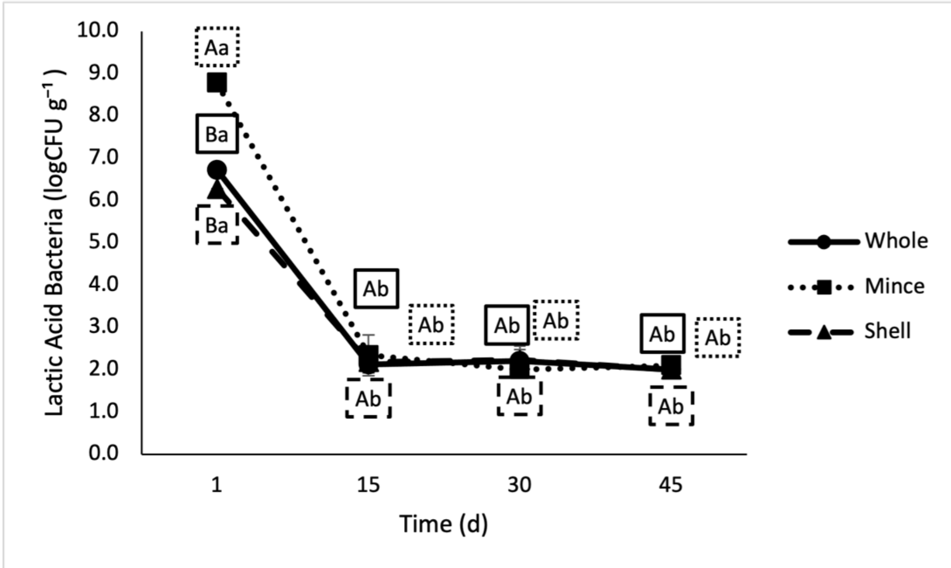

Figure A.3. Lactic Acid Bacterial Population of Crab Sauces Fermented from Separated Streams Over Time. The lowercase letters designate significant differences over time. The uppercase letters designate significant differences among treatments. The error bars represent the standard deviation. (n=3)

The *Bacillus* bacterial population (Figure A.4.) significantly decreased after day one from an average of 3.0 ± 0.0 logCFU g^{-1} to 2.1 ± 0.1 logCFU g^{-1}. This followed a similar, but less drastic pattern as seen in the proteolytic, lactic acid bacteria, and total plate count bacterial populations, indicating the populations being observed had a similar reaction to high salt content.

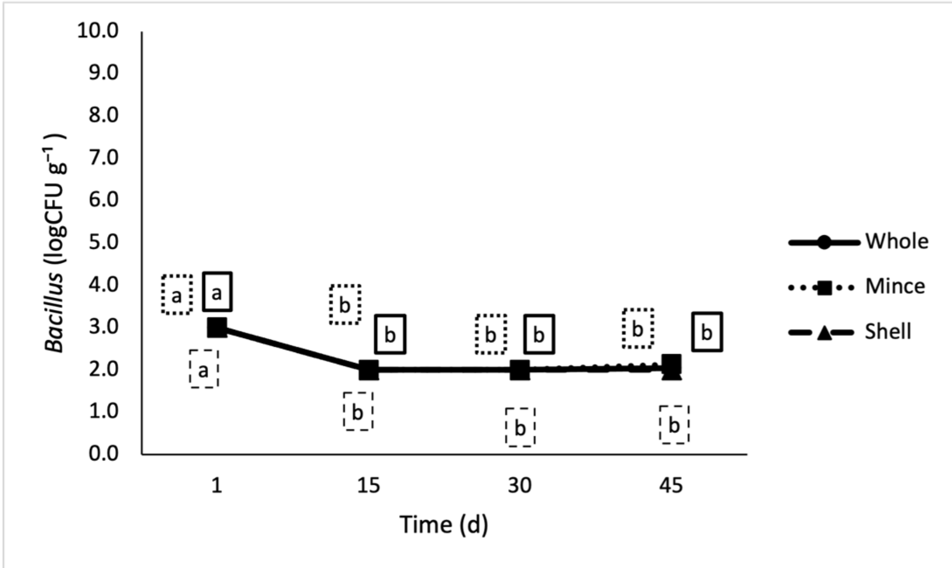

Figure A.4. *Bacillus* Population of Crab Sauces Fermented from Separated Streams Over Time. The lowercase letters designate significant differences over time. The error bars represent the standard deviation. (n=3)

There was a steady increase in pH (Figure A.5.) over time from an average of 7.47 ± 0.15 on day 15 to 8.07 ± 0.09 on day 45 among all treatments.

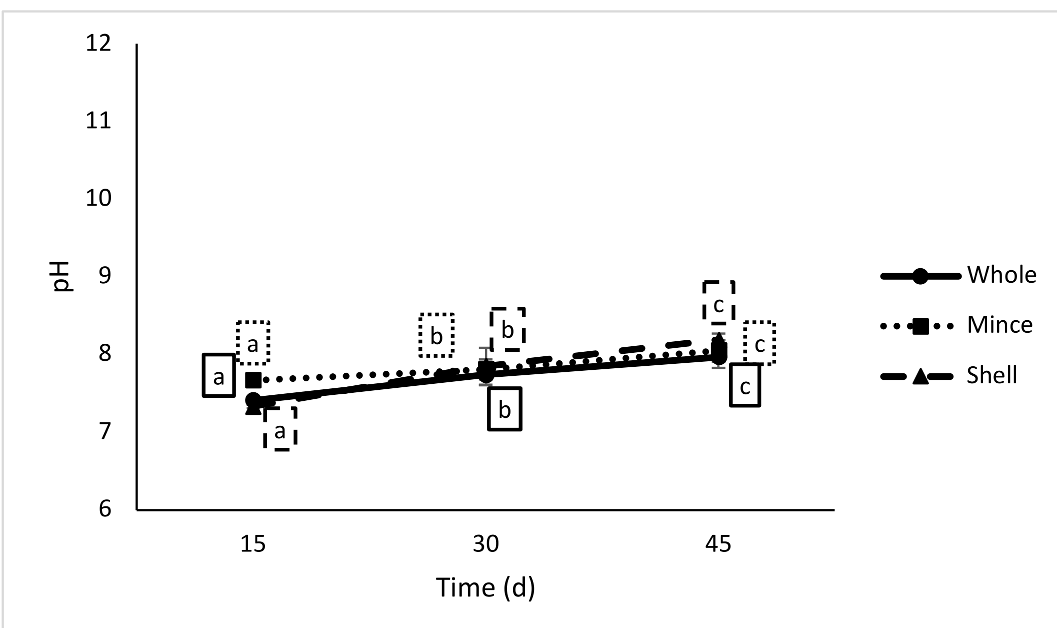

Figure A.5. pH of Crab Sauces Fermented from Separated Streams Over Time. The lowercase letters designate significant differences over time. (n=3)

Crab sauce made with crab mince (0.741 ± 0.005) and whole crab (0.738 ± 0.002) had a significantly lower water activity (Table A.1.) than the sauce fermented from the shell starting material (0.766 ± 0.009 Aw) at all time points throughout the fermentation.

Table A.1. Water Activity in Lab Fermented Crab Sauce Samples

Time (d)	**Whole** (Aw ± sd)	**Mince** (Aw ± sd)	**Shell** (Aw ± sd)
15	0.740 ± 0.007 A	0.743 ± 0.004 A	0.753 ± 0.001 B
30	0.734 ± 0.007 A	0.747 ± 0.009 A	0.773 ± 0.006 B
45	0.738 ± 0.002 A	0.736 ± 0.005 A	0.733 ± 0.000 B

The capital letters designate significant differences among treatments. (n=3)

In this study, the sauce fermented from the crab shell appeared less appealing, as seen in Figure A.6. Not only was the sauce not appealing but it was not present in all samples.

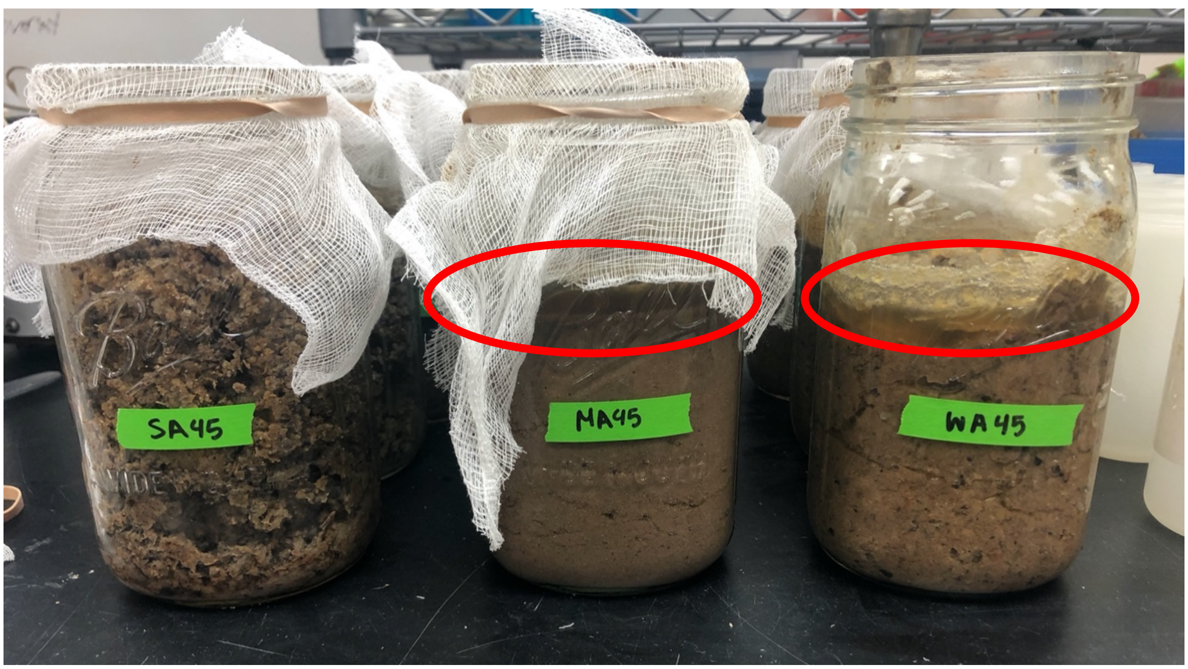

Figure A.6. Day 45 Fermented Samples. As seen in both the red circles in the picture above, the fermented whole crab (WA45) and the fermented crab mince (MA45) have layers of fermented crab sauce near the top of the jar. However, the fermented crab shell (SA45) has no fermented crab sauce layer, resulting in a non-viable product.

Since the crab sauce fermented from the shell did not produce a viable product, these fermented samples were not tested for amine nitrogen. The amine nitrogen (Figure A.7) significantly increased over time from an average of 384.42 ± 9.92 mg $100mL^{-1}$ to an average of 603.75 ± 35.00 mg $100mL^{-1}$.

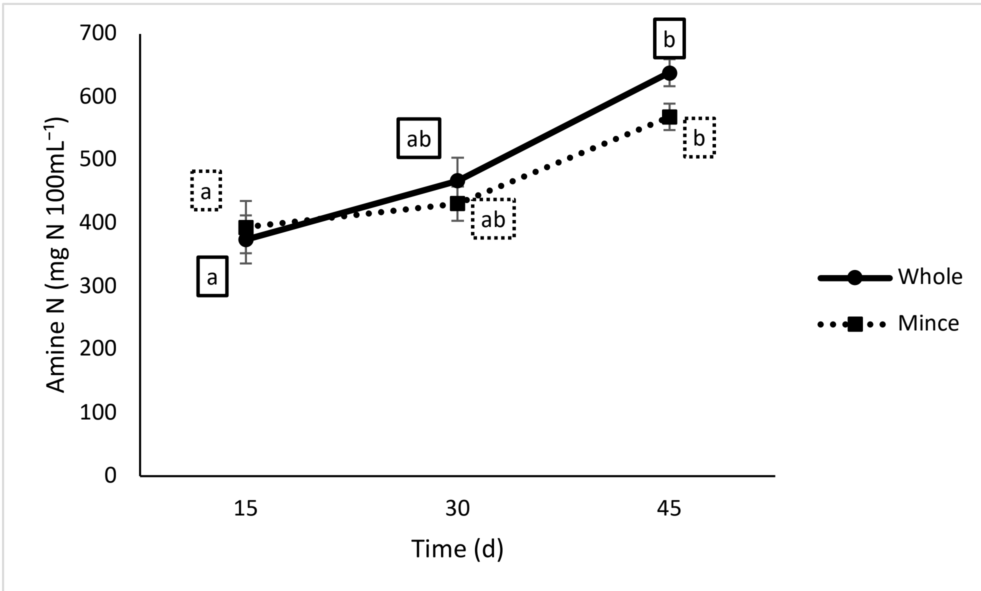

Figure A.7. Amine Nitrogen of Crab Sauces Fermented from Separated Streams Over Time. The lowercase letters designate significant differences over time. The error bars represent the standard deviation. (n=3)

There were no distinct trends in total biogenic amine content according to crab stream (Table A.2.). The average total biogenic amine content was 8.69 ± 2.39 mg $100mL^{-1}$. There was a significant increase in the total biogenic amines on day 30 and 45 for the whole crab only. There was an almost significant difference between whole crab on day 0 and 45 (p-value of 0.053).

Table A.2. Total Biogenic Amines in Lab Fermented Crab Sauces							
Starting Material	Time (d)	Histamine	Agmatine	Putrescine	Cadaverine	Tyramine	Total Biogenic Amines
Whole	1	2.28 ± 0.00 B	0.26 ± 0.00	N.D.	0.52 ± 0.00 Ba	3.37 ± 0.00	6.42[b]
	15	6.19 ± 0.63	1.14 ± 0.25	1.89 ± 0.37	0.23 ± 0.03 b	N.D.	9.07
	30	4.43 ± 0.40	1.44 ± 0.53	2.17 ± 0.71	0.22 ± 0.02 b	2.00 ± 0.00	8.21a
	45	4.50 ± 0.27	3.28 ± 1.62	3.32 ± 0.86	0.29 ± 0.00 b	2.79 ± 0.51	13.25 b[b]
Mince	1	1.93 ± 0.00A	0.63 ± 0.00	N.D.	0.24 ± 0.00 A	3.37 ± 0.00	6.17
	15	3.81 ± 0.07	1.82 ± 0.00	2.30 ± 0.56	0.23 ± 0.07	1.93 ± 0.00	7.58
	30	4.97 ± 0.60	1.82 ± 0.31	3.10 ± 0.66 A	0.18 ± 0.02	N.D.	10.07
	45	4.32 ± 0.40	1.57 ± 0.35	3.49 ± 0.32	0.28 ± 0.07	N.D.	9.65
Shell	1	1.76 ± 0.00 a	0.61 + 0.00	N.D.	0.18 ± 0.00 A	2.32 ± 0.00	4.87
	15	4.97 ± 0.45 b	1.37 ± 0.00	1.37 ± 0.25	0.22 ± 0.01	N.D.	7.25
	30	4.13 ± 0.50 ab	1.09 ± 0.17	0.63 ± 0.52 B	0.26 ± 0.00	2.00 ± 0.13	7.98
	45	4.34 ± 0.76 ab	1.30 ± 0.03	2.23 ± 0.45	0.23 ± 0.01	1.96 ± 0.00	9.08

Uppercase letters designate significant differences between treatments within the same timepoint. Lowercase letters designate significant differences between timepoints within treatment. (n=3)
[b]Almost significantly different

A.3. Separation of Streams Conclusion

All of the bacterial populations decreased significantly after the first day of fermentation. After the first day of fermentation there were no significant differences, showing no benefit to separating streams. The pH and the amine nitrogen significantly increased over time due to fermentation time, but not the separation of streams. The water activity was significantly higher in the crab sauce fermented from the shell, but there were no significant differences between the crab sauce fermented from the whole crab and the mince. The histamine concentration was below the legally acceptable limit of 50mg $100mL^{-1}$ (Food and Drug Administration, 2019). In conclusion, the separation of streams is not a recommended course of action for the fermentation of green crab due to the additional equipment that would be needed to complete this and the increased cost this would incur for no significant differences between the crab sauce produced.

A.4. *Staphylococcus carnosus* Starter Culture Results

The proteolytic bacterial population (Figure A.8.) decreased over time in both inoculated and uninoculated sauces. There was a peak in the proteolytic bacterial population in the crab sauce with no starter culture on day 60. The proteolytic bacterial population was significantly higher in the crab sauce with *S. carnosus* added as a starter culture than the crab sauce with no culture (6.1 ± 0.0 logCFU g^{-1} and 2.9 ± 0.0 logCFU g^{-1} respectively) on the original day of mixing before fermentation, due to the presence of the starter culture itself. There were no significant differences between the crab sauce fermented with no culture and the crab sauce fermented with *S. carnosus* on day 90, resulting in an average proteolytic bacterial population of 2.0 ± 0.0 logCFU g^{-1} regardless of treatment.

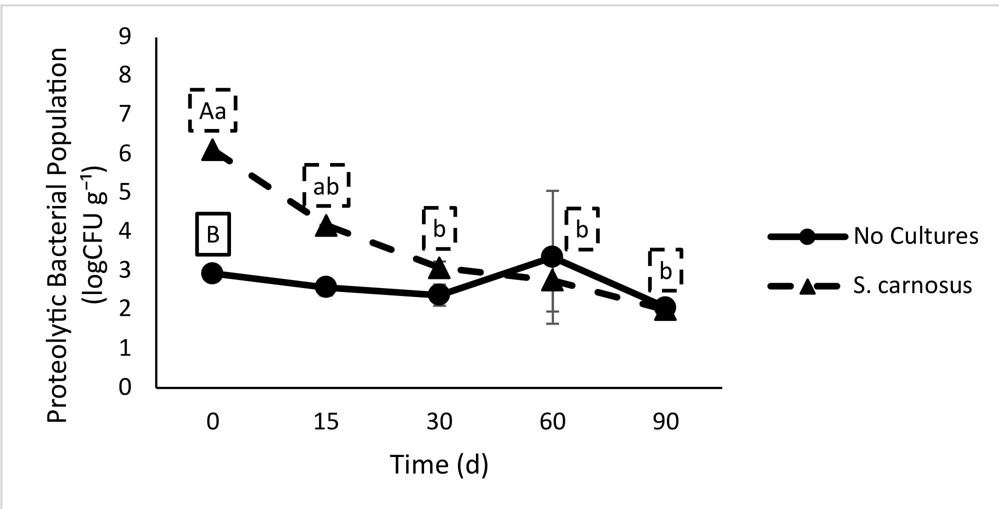

Figure A.8. Proteolytic Bacterial Population of Crab Sauces Fermented at 37°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The histamine forming bacterial population (**Figure A.9**) was very similar to the proteolytic bacterial population. The population of histamine forming bacteria found in the crab sauce fermented with *S. carnosus* as a starter culture showed a decreasing trend over time. The histamine forming bacterial population was significantly higher in the crab sauce with *S. carnosus* as a starter culture than the crab sauce with no culture (6.2 ± 0.0 logCFU g^{-1} and 2.7 ± 0.0 logCFU g^{-1} respectively) on the original day of mixing before fermentation. The crab sauce with no culture, like with the proteolytic bacterial population, peaked on day 60.

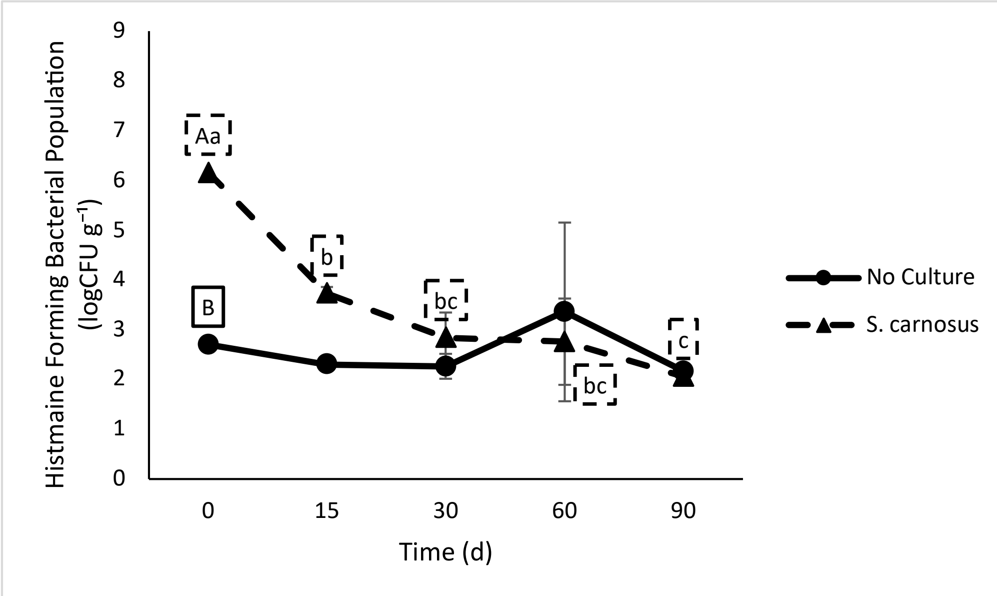

Figure A.9. Histamine Forming Bacterial Population of Crab Sauces Fermented at 37°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The mannitol fermenting staphylococci population (Figure A.10.) follows a very similar trend as the proteolytic and histamine forming bacterial populations. The mannitol fermenting staphylococci population in the crab sauce fermented with *S. carnosus* as a starter culture decreased after day 0 and did not change maintaining an average population of 2.2 ± 0.1 logCFU g^{-1} on all other timepoints of the fermentation. The mannitol fermenting staphylococci bacterial population was significantly higher in the crab sauce fermented with *S. carnosus* as a starter culture than the crab sauce fermented with no starter culture (6.2 ± 0.0 logCFU g^{-1} and 2.8 ± 0.0 logCFU g^{-1} respectively) on the original day of mixing before fermentation, but the culture appeared to experience nearly complete inactivation by d 15 of fermentation.

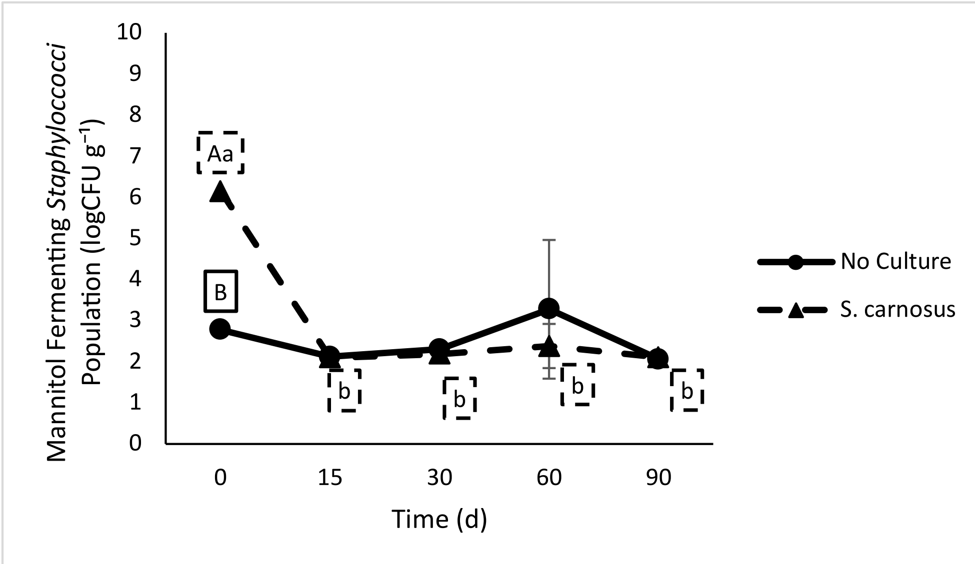

Figure A.10. Mannitol Fermenting Staphylococci Bacterial Population of Crab Sauces Fermented at 37°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The lactic acid bacterial population (Figure A.11.) follows almost the same trend that all of the other microbial populations (proteolytic, histamine forming, and mannitol fermenting staphylococci) indicating that these are probably looking at the same population of bacteria. The lactic acid bacterial population decreases over the fermentation time. On day 0, after the crab, salt, and starter cultures had been mixed, the crab mixture containing *S. carnosus* as a starter culture was 6.12 ±0.0 logCFU g^{-1}, significantly higher than the crab mixture with no starter culture which was 2.6 ± 0.0 logCFU g^{-1}.

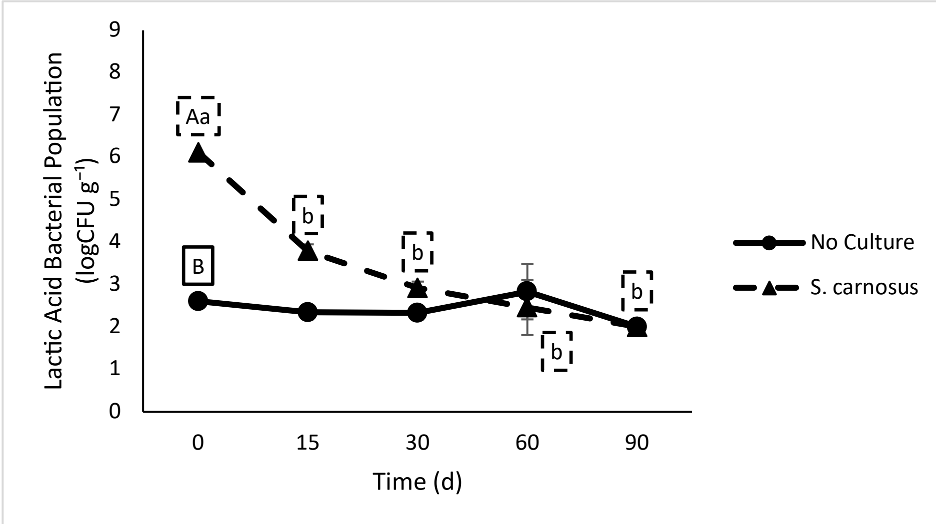

Figure A.11. Lactic Acid Bacterial Population of Crab Sauces Fermented at 37°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The pH (Figure A.12.) increased and then slightly decreased for both the crab sauce fermented with *S. carnosus* and with no starter culture. There were no significant differences due to the presence of *S. carnosus* as a starter culture. The pH of the crab sauce fermented with *S. carnosus* was higher and peaked at 7.8 ± 0.1 on day 60.

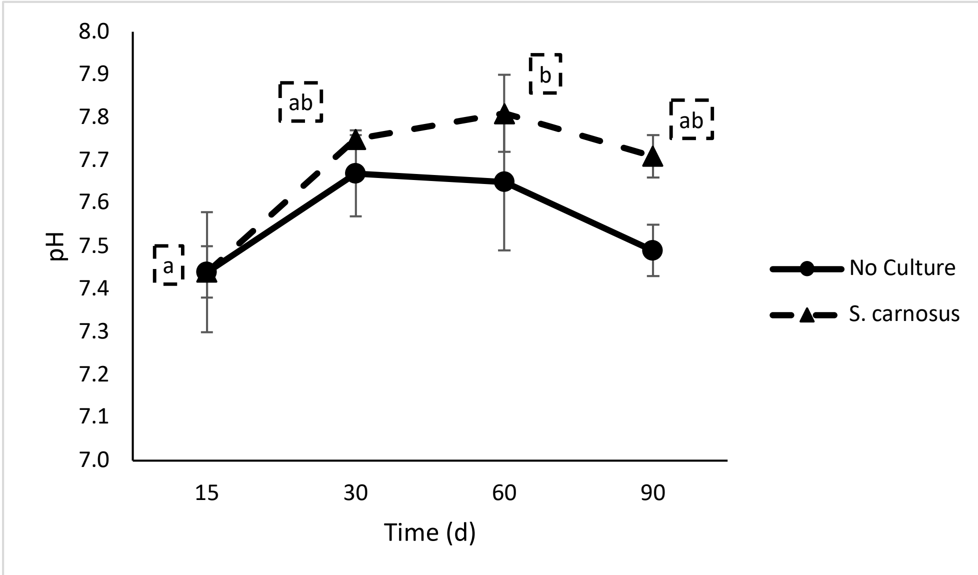

Figure A.12. pH of crab Sauces Fermented at 37°C. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The water activity (Table A.3.) of the crab sauce fermented with *S. carnosus* peaked on day 90 at 0.746 ± 0.008. The water activity of the crab sauce fermented with no starter cultures had a slightly positive trend but with no significant differences. The average water activity of the crab sauce without starter cultures was 0.737 ± 0.002.

Table A.3. Water Activity of Crab Sauces Fermented at 37°C		
Time (d)	No Culture	*S. carnosus*
15	0.737 ± 0.004	0.732 ± 0.001
30	0.734 ± 0.003	0.731 ± 0.002
60	0.738 ± 0.007	0.731 ± 0.001
90	0.737 ± 0.009	0.746 ± 0.008

No significant differences were identified. (n=3)

The non-enzymatic browning (Table A.4.) of both the crab sauce fermented with *S. carnosus* and without a starter culture followed a positive trend throughout the fermentation. There were no significant differences throughout the course of the fermentation due to time or treatment. The non-enzymatic browning averaged 0.26 ± 0.07 throughout the course of the fermentation for both treatments.

Table A.4. Non-enzymatic Browning of Crab Sauces Fermented at 37°C		
Time (d)	No Culture	*S. carnosus*
15	0.223 ± 0.034	0.145 ± 0.055
30	0.225 ± 0.087	0.199 ± 0.061
60	0.366 ± 0.060	0.331 ± 0.126
90	0.253 ± 0.133	0.302 ± 0.145

No significant differences were identified. (n=3)

The amine nitrogen (Figure A.13.) of the lab-fermented crab sauces both with and without *S. carnosus* followed a positive trend over time. There were no significant differences due to treatment. The amine nitrogen increased from an average of 509.06 ± 47.06 mgN $100mL^{-1}$ on day 15 to an average of 658.39 ± 21.39 mgN $100mL^{-1}$ on day 90.

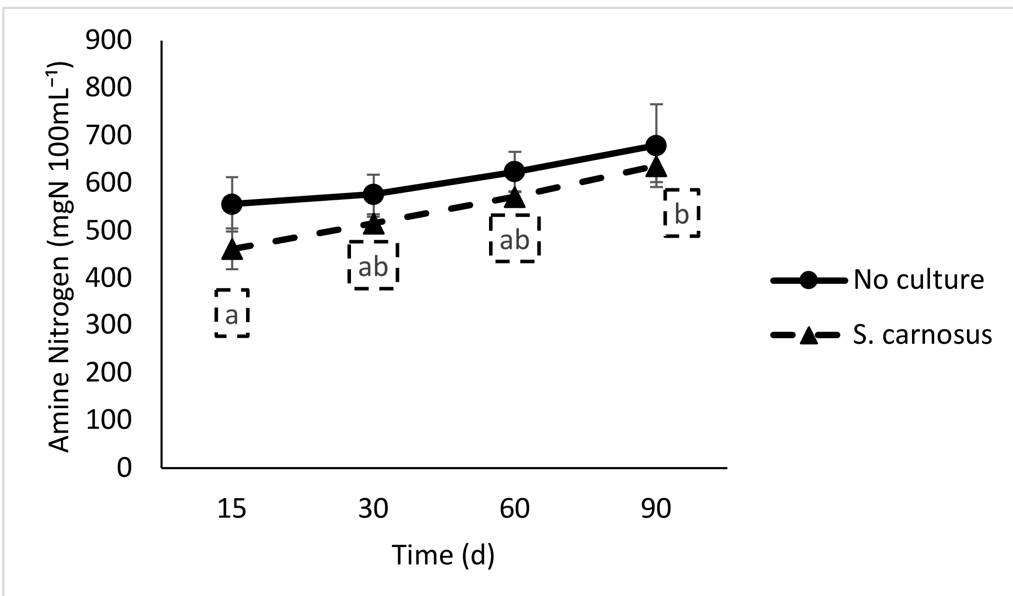

Figure A.13. Amine Nitrogen of Crab Sauces Fermented at 37°C. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

Of the biogenic amines (Table A.5.) quantified, histamine, agmatine, and the total biogenic amines had model level effects from day but not presence of starter culture. Tyramine had a model level effect due to treatment. Cadaverine was not recovered at either treatment or any time points. Putrescine was only identified in the pre-fermented crabs. All of the histamine measures were below the legally allowed limit of 50mg $100mL^{-1}$ (Food and Drug Administration, 2019).

Table A.5. Total Biogenic Amines in Crab Sauces Fermented at 37°C							
Treatment	Time (d)	Histamine (mgN 100mL^{-1})	Agmatine (mgN 100mL^{-1})	Putrescine (mgN 100mL^{-1})	Cadaverine (mgN 100mL^{-1})	Tyramine (mgN 100mL^{-1})	Total Biogenic Amines
No Starter Culture	0	3.23 ± 0.36 a	2.04 ± 0.16	4.14 ± 0.05	N.D.	2.10 ± 0.10 *	11.51
	15	7.03 ± 0.75 ab	5.23 ± 1.30	N.D.	N.D.	2.10 ± 0.20 *	14.36
	30	8.74 ± 0.34 b	5.15 ± 0.78	N.D.	N.D.	2.18 ± 0.14 *	16.07
	60	7.91 ± 3.01 b	4.20 ± 0.92	N.D.	N.D.	2.79 ± 0.51 *	14.90
	90	8.22 ± 0.64 b	4.76 ± 0.78	N.D.	N.D.	2.25 ± 0.25 *	15.23
S. carnosus	0	3.23 ± 0.36 a	2.04 ± 0.16 a	4.14 ± 0.05	N.D.	2.10 ± 0.10 *	11.51
	15	7.28 ± 1.04 b	7.36 ± 1.89 b	N.D.	N.D.	2.81 ± 0.33 *	17.45
	30	8.20 ± 0.65 b	4.84 ± 0.26 ab	N.D.	N.D.	2.34 ± 0.24 *	15.38
	60	8.61 ± 0.82 b	4.88 ± 1.21 ab	N.D.	N.D.	2.48 ± 0.34 *	15.97
	90	9.04 ± 0.89 b	4.93 ± 0.49 ab	N.D.	N.D.	2.69 ± 0.06 *	16.66

Lowercase letters designate significant differences across time within treatment.

*Designates significant differences due to treatment

A.5. *Tetragenococcus halophilus* Starter Culture Results

The proteolytic bacterial population (Figure A.14.) decreased throughout the fermentation for the crab sauce fermented with *T. halophilus* as a starter culture. The crab sauce fermented without a starter culture maintained a low proteolytic bacterial population except for on day 15, where the population peaked at 8.5 ± 0.0 logCFU g^{-1}. Although there were significant differences due to treatment at the beginning of the fermentation, there were no significant differences due to treatment after day 30, resulting in an average proteolytic bacterial population of 2.3 ± 0.2 logCFU g^{-1} on day 90.

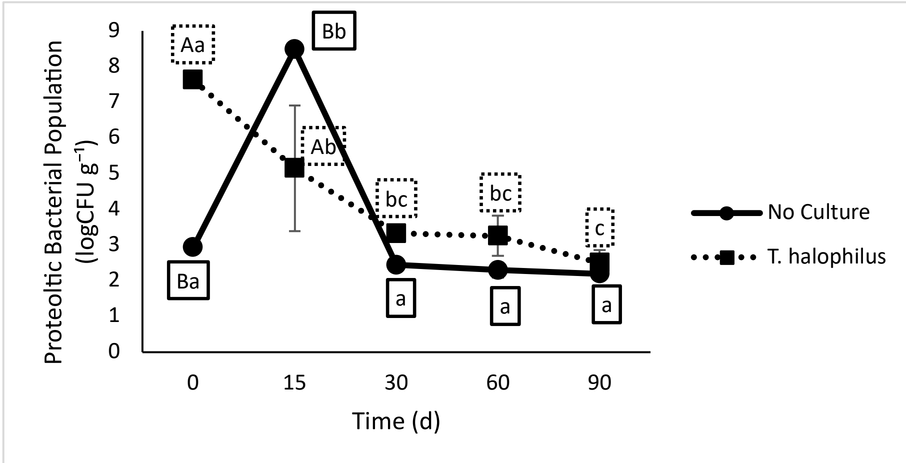

Figure A.14. Proteolytic Bacterial Population of Crab Sauces Fermented at 30°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The histamine forming bacterial population (Figure A.15.) followed very similar trends as the proteolytic bacteria. The histamine forming bacterial population followed a downward trend throughout the fermentation for the crab sauce that was fermented using *T. halophilus* as a starter culture. The histamine forming bacterial population for the crab sauce fermented without a starter culture peaked on day 15 at 8.5 ± 0.0 logCFU g^{-1}. On day 90, there were no significant differences due to treatment resulting in an average histamine forming bacterial population of 2.3 ± 0.3 logCFU g^{-1}.

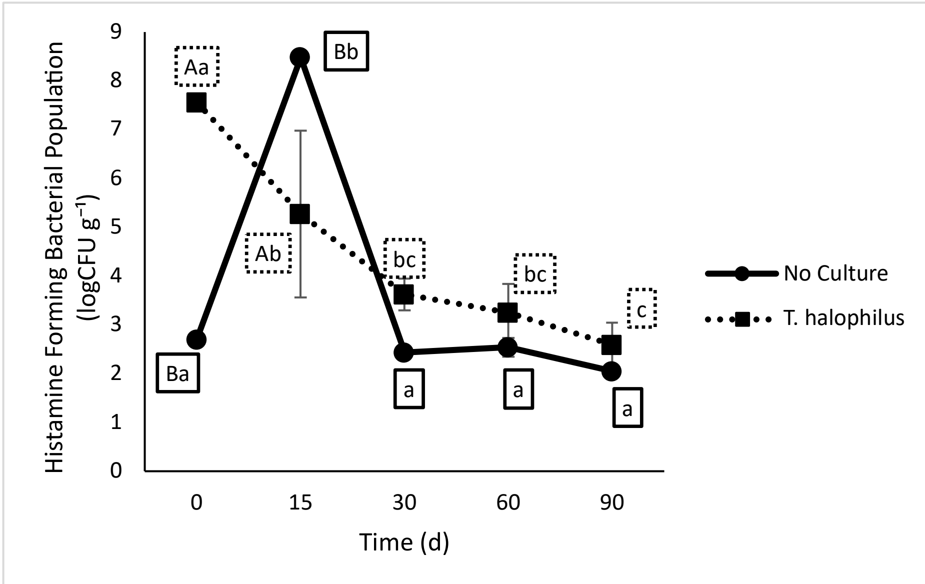

Figure A.15. Histamine Forming Bacterial Population of Crab Sauces Fermented at 30°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The mannitol fermenting *Staphylococci* bacterial population (Figure A.16) closely mirrored that of the proteolytic and lactic acid bacterial populations. The population decreased over the course of the fermentation in the crab sauce that was fermented with *T. halophilus* as a starter culture. The crab sauce fermented without a starter culture significantly peaked at 9.2 ± 0.3 logCFU g^{-1} on day 15. There were no significant differences due to treatment on day 90, resulting in an average population of 2.1 ± 0.1 logCFU g^{-1}.

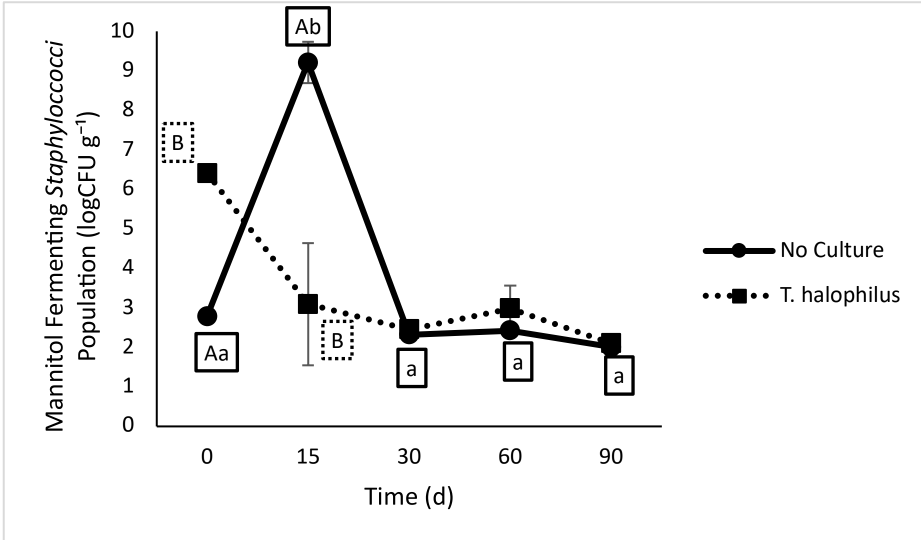

Figure A.16. Mannitol fermenting *Staphylococci* Bacterial Population of Crab Sauces Fermented at 30°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The lactic acid bacterial population (Figure A.17.) followed a trend almost identical to the other bacteria (proteolytic, histamine forming, and mannitol fermenting *Staphylococci*) indicating the likelihood that all of these measures are looking at the same bacterial population. The crab sauce fermented with *T. halophilus* as a starter culture significantly decreased over the course of the fermentation from 7.5 ± 0.0 logCFU g^{-1} to 2.9 ± 0.2 logCFU g^{-1}. The crab sauce fermented with no starter culture peaked at 8.5 ± 0.0 logCFU g^{-1} on day 15. There were no significant differences due to treatment on day 90, resulting in an average bacterial population of 2.5 ± 0.4 logCFU g^{-1}.

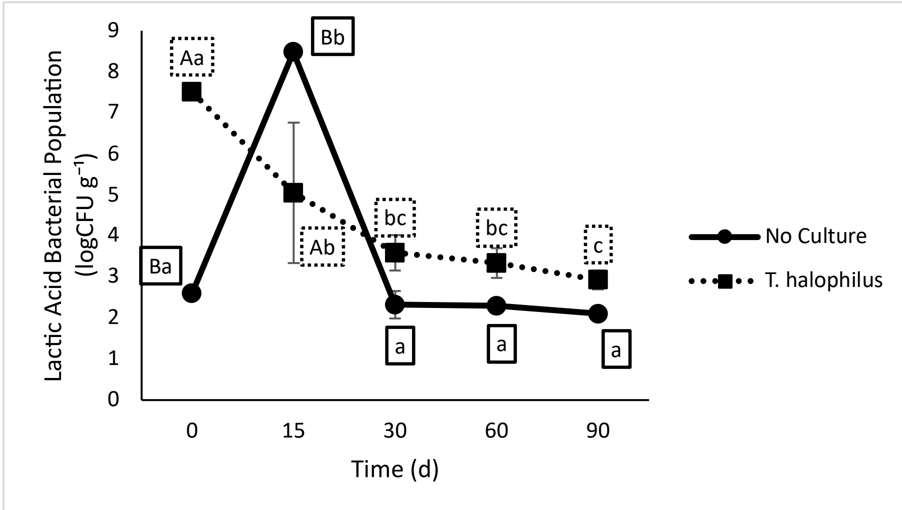

Figure A.17. Lactic Acid Bacterial Population of Crab Sauces Fermented at 30°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

The pH (Figure A.18.) of the crab sauce fermented with *T. halophilus* as a starter culture showed a positive trend over the course of the fermentation with a peak of 7.91 ± 0.02 on day 60. The pH of the crab sauce fermented with no starter culture showed a negative trend over the course of the fermentation, resulting in a pH of 7.56 ± 0.05 on day 90.

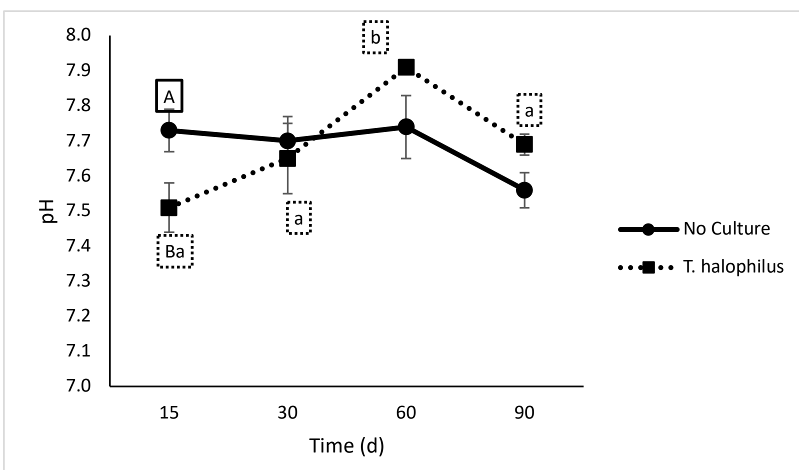

Figure A.18. pH of Crab Sauces Fermented at 30°C. Uppercase letters designate significant differences between treatments on individual sampling days. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

There were no significant differences in water activity (Figure A.19) due to treatment. The water activity of the crab sauce fermented with *T. halophilus* peaked at 0.751 ± 0.004 on day 90. The crab sauce fermented without a starter culture showed a slightly positive trend with no significant differences over time, showing an average water activity of 0.738 ± 0.004.

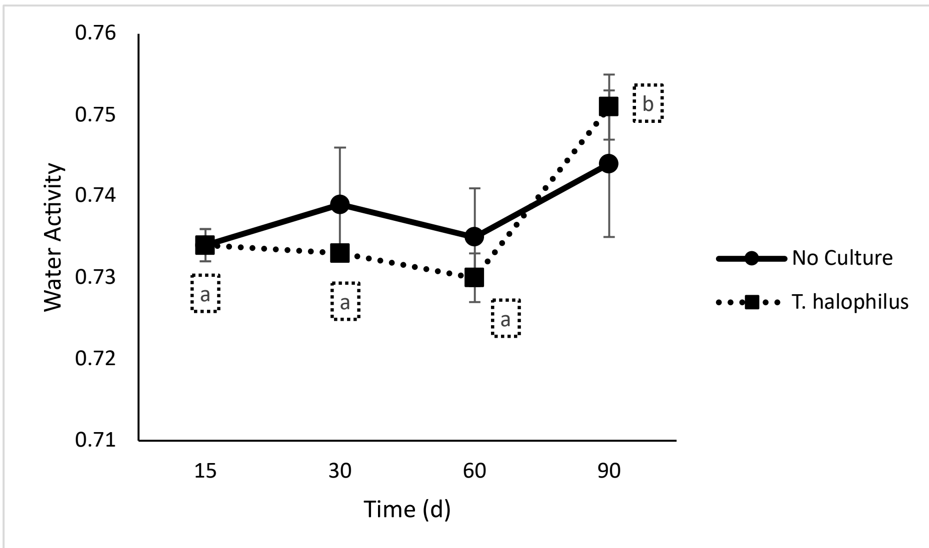

Figure A.19. Water Activity of Crab Sauces Fermented at 30°C. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

There were no significant differences in non-enzymatic browning (Table A.6) due to time or treatment. Both treatments showed a slightly positive trend over the course of the fermentation. Both treatments peaked on day 60 with an average reading of 0.265 ± 0.020.

Table A.6. Non-Enzymatic Browning of Crab Sauces Fermented at 30°C

Time (d)	No Culture	*T. halophilus*
15	0.242 ± 0.010	0.197 ± 0.011
30	0.226 ± 0.020	0.218 ± 0.048
60	0.284 ± 0.050	0.245 ± 0.069
90	0.269 ± 0.078	0.234 ± 0.067

No significant differences were identified. (n=3)

The amine nitrogen (Figure A.20.) showed a positive trend over the course of the fermentation for both treatments. The amine nitrogen of the crab sauce fermented with *T. halophilus* as a starter culture significantly increased over the course of the fermentation from 475.22 ± 29.16 mgN $100mL^{-1}$ on day 15 to 653.33 ± 40.32 mgN 100mL^{-1} on day 90. There were no significant differences due to treatment, resulting in an average amine nitrogen of 668.11 ± 14.78 mgN 100mL^{-1} on day 90.

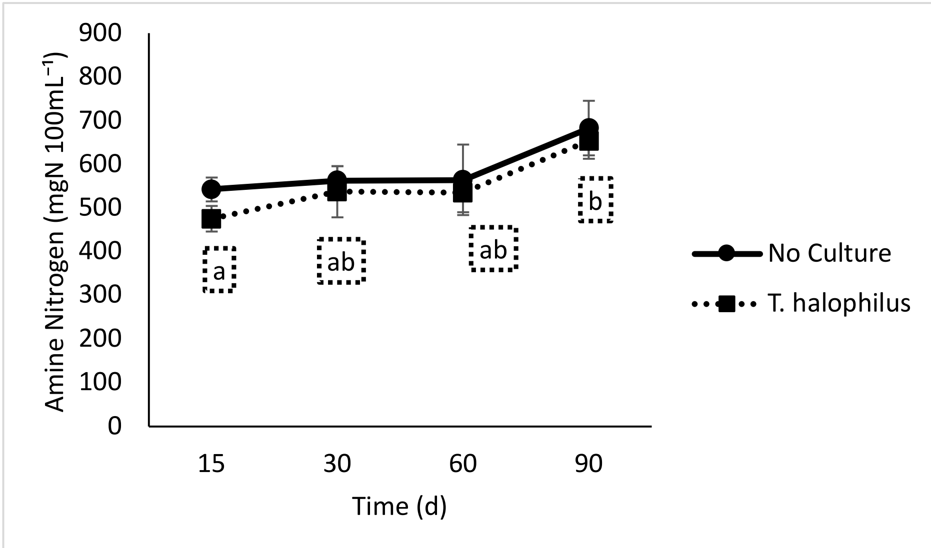

Figure A.20. Amine Nitrogen of Crab Sauces Fermented at 30°C. Lowercase letters designate significant difference within treatment across time. The error bars represent the standard deviation. (n=3)

There were significant model level effects in the biogenic amines (Table A.7.) from the fermentation time for the histamine and agmatine. The total biogenic amines had model level effects from the fermentation time and the treatment. Cadaverine was not identified in any of the samples. Putrescine was only identified in the pre-fermented material and in the crab sauce fermented without starter cultures on day 15. All of the histamine levels were below the legally allowed limit of 50mg 100mL^{-1} (Food and Drug Administration, 2019).

Table A.7. Total Biogenic Amines in Crab Sauces Fermented at 30°C							
Treatment	Time (d)	Histamine (mgN 100mL^{-1})	Agmatine (mgN 100mL^{-1})	Putrescine (mgN 100mL^{-1})	Cadaverine (mgN 100mL^{-1})	Tyramine (mgN 100mL^{-1})	Total Biogenic Amines
No Starter Culture	0	3.23 ± 0.36 a	2.04 ± 0.16 a	4.14 ± 0.05	N.D.	2.10 ± 0.10	11.51 a*
	15	7.29 ± 0.98 ab	3.84 ± 0.25 ab	5.58 ± 0.92	N.D.	N.D.	16.71 ab*
	30	10.37 ± 1.16 b	5.97 ± 0.18 b	N.D.	N.D.	2.32 ± 0.30	18.66 b*
	60	7.85 ± 1.91 b	4.29 ± 0.64 ab	N.D.	N.D.	2.40 ± 0.39	14.54 ab*
	90	8.57 ± 0.99 b	4.93 ± 0.14 b	N.D.	N.D.	2.63 ± 0.17	16.13 ab*
T. halophilus	0	3.23 ± 0.36 a	2.04 ± 0.16 a	4.14 ± 0.05	N.D.	2.10 ± 0.10	11.51*
	15	8.50 ± 0.35 b	5.05 ± 1.00 b	N.D.	N.D.	2.23 ± 0.13	15.78*
	30	8.31 ± 1.03 b	4.69 ± 0.32 b	N.D.	N.D.	2.34 ± 0.10	15.34*
	60	5.17 ± 2.80 ab	5.08 ± 1.25 b	N.D.	N.D.	2.38 ± 0.48	12.63*
	90	7.80 ± 0.67 b	3.63 ± 0.01 ab	N.D.	N.D.	2.29 ± 0.49	13.72*

Lowercase letters designate significant differences across time within treatment.
*Designates significant differences due to treatment

A.6. Starter Culture Conclusion

With the inclusion of these starter cultures was the expectation of a lowered pH and a lowered histamine content. However, this was not demonstrated for either starter culture. With the inclusion of *S. carnosus* and *T. halophilus* there were no advantages to including the starter cultures. The pH of the crab sauce that was fermented with *S. carnosus* increased when expected to decrease throughout the course of the fermentation. This method is not recommended due to the lack of advantages from including the culture and the additional cost of including either starter culture.

A.7. References

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BIOGRAPHY OF THE AUTHOR

Delaney Greiner was born in Cincinnati, Ohio on December 27, 1996. She was raised in Cincinnati, Ohio and graduated from Mother of Mercy High School in 2015. She attended Clemson University and graduated in 2019 with a bachelor's degree in Food Science and Human Nutrition, graduating cum laude. After receiving her master's degree from the University of Maine, Delaney will be moving to Florida and joining American Sugar Refining, an international sugar-refining company, as an Associate Food Scientist II on a team focused on new product development to begin her career in the field of food science. Delaney is a candidate for the Master of Science degree in Food Science and Human Nutrition from the University of Maine in August 2021.